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Development of a Versatile Antibody Cloning and Expression System

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Development of a Versatile Antibody Cloning and Expression System

by

Tihomir Dodev

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in King's College London

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ABSTRACT

Over the last three decades, recombinant monoclonal antibodies (mAbs) have become the key tool for basic research, diagnosis and treatment of human diseases. This has required the selection of recombinant antibodies with high affinity for appropriate epitopes on the target antigen and other desirable characteristics, such as their isotype and effector functions. In Biopharmaceutical communities specialised in antibody production, expression levels of grams per litre have been reached. However, lack of a suitable manufacturing platform, which ensures consistent antibody production, has always been one of the major impediments to the development of recombinant antibody material in academia. To overcome this barrier, we have developed a unique cloning method for one-step assembly of antibody heavy- and light-chain DNAs in a single mammalian expression vector. The DNA fragments assembled in this system do not rely on restriction enzyme- and ligase-dependant methods, thus minimising the steps involved in the cloning procedure. This allows the reproducible generation of fully functional recombinant antibodies of any species and isotype with any desired specificity. In less than four weeks, tens of milligrams per litre can be achieved, from cloning through to harvesting of transfected cell supernatants, providing an unbiased manufacturing platform compared to the currently available antibody expression methods in academia. The system proved to be very efficient and readily adaptable for the high-throughput screening of melanoma patient-derived antibody candidates with clinical potential. It enabled the parallel comparative functional studies between IgE and IgG1 isotypes in an *in vivo* xenograft model of melanoma. The IgE isotype showed superior efficacy in restricting tumour growth, which encouraged us to continue developing antibody discovery methods, and pursuing melanoma antigen-specific antibodies as future effective therapies of this disease.

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ABBREVIATIONS

APC	Antigen Presenting Cell
BCR	B-cell Receptor
bp	Base pairs of nucleic acids
CDR	Complementarity Determining Region
C_H	Heavy Chain Constant Domain
CHO	Chinese hamster ovarian cells
COS	*Cercopithecus aethiops* kidney cells
Cκ/λ	Kappa / Lambda Chain Constant Domain
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribonucleic acid
FBS	Foetal Bovine Serum
FCS	Fetal calf serum
FcγR	Receptors for the Fc region of IgG
FcεRI	High affinity Fc receptor for IgE
FITC	Fluorescein Isothiocyanate
FWR	Framework Region
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
HEK293	Human embryonic kidney 293 cells
Ig	Immunoglobulin
mAb	Monoclonal antibody
NGS	Normal Goat Serum
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBS-T	Phosphate buffered saline (with Tween 20)
PCR	Polymerase Chain Reaction

PEI	Polyethylenimine
PIPE	Polymerase Incomplete Primer Extension
RBL	Rat basophil-like cells
RNA	Ribonucleic acid
RT	Reverse Transcription / Transcriptase
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
TCR	T-cell Receptor
UV	Ultraviolet light
V(D)J	Variable, diversity, joining region of immunoglobulin
V_H	Heavy Chain Variable Domain
Vκ	Light Chain Variable Domain

1 INTRODUCTION

1.1 Antibodies

In 1890 Behring and Kitasato discovered the presence of "something in the blood that was able to neutralize diphtheria toxin": transfusing sera already containing the antitoxin activity would protect the recipient animal from infection with the same toxin (Behring E, 1890). This was further elucidated when Paul Ehrlich described the interaction between the antitoxin and diphtheria toxin, introducing the term "antibody" in 1891 (Ehrlich, 1891).

Today, it is very well known that the immune system is responsible for protecting organisms from harmful infections. The adaptive immune response has evolved to recognise various antigens associated with infectious agents and initiate appropriate cellular activities to repress the infectious threat. The molecules responsible for antigen recognition are T-cell receptors (TCR) on the surface of T-cells, and B-cell receptors (BCR) on the surface of B-cells. B-cell receptors are secreted as antibodies following differentiation of B-cells into plasma cells. Antibodies are remarkably diverse, not only do they recognise millions of antigens, but also each class of antibody has a different effector function. TCR antigen binding requires the presentation of antigenic peptides by Major Histocompatibility Complex (MHC) molecules present on the surface of Antigen Presenting Cells (APC) such as B-cells, dendritic cells and Langerhans cells. B-cell receptors (also known as Immunoglobulins) and antibodies can recognise antigen in the absence of facilitator cells. Antigen binding to BCRs may result in internalisation and processing for subsequent antigen presentation to TCRs. Secreted antibodies

couple antigen to immune effector cells via antibody-specific cell surface receptors (Janeway et al, 2001)

1.2 Antibody structure and function

Antibodies are produced by B lymphocytes by random combination of V and J (light chain), and V, D, and J (heavy chain) regions. Each antibody is a polypeptide ligand composed of two identical heavy (H) and two identical light (L) chains, interconnected by disulfide bonds. Each of the heavy and light chains can be subdivided into Constant (C) and Variable (V) immunoglobulin domains (Figure 1.1). The light chain may be one of two isotypes, κ or λ, and is comprised of a single Cκ or Cλ domain plus a corresponding Vκ or Vλ domain. The heavy chain C (C_H) domains determine the antibody class: Cε for IgE, Cμ for IgM, Cδ for IgD, Cγ for IgG and Cα for IgA. All heavy chains are comprised of a single variable (V_H) domain and four C_H domains in the case of IgE and IgM, or three C domains in IgD, IgG and IgA (Figure 1.2). The Fc fragment, comprising C_H domains 2, 3 (and 4) of an antibody (Figure 1.2), is responsible for binding to specific antibody receptors on the surface of effector cells. As different cell types express receptors with different isotype specificity, the Fc fragment determines the antibody effector function. The V domains of the heavy and light chain participate in antigen recognition and therefore require regions of hypervariability from one antibody to another, in order to recognise and bind with a high degree of specificity and affinity to the vast plethora of antigens to which the immune system is continuously exposed. Within the antibody structure these regions are localised into six hypervariable loops, or complementarity determining regions (CDR), three from V_H and three from V_L (Figure 1.1), which in combination form the antigen binding site of the Fab fragment of the antibody (Janeway et al, 2001).

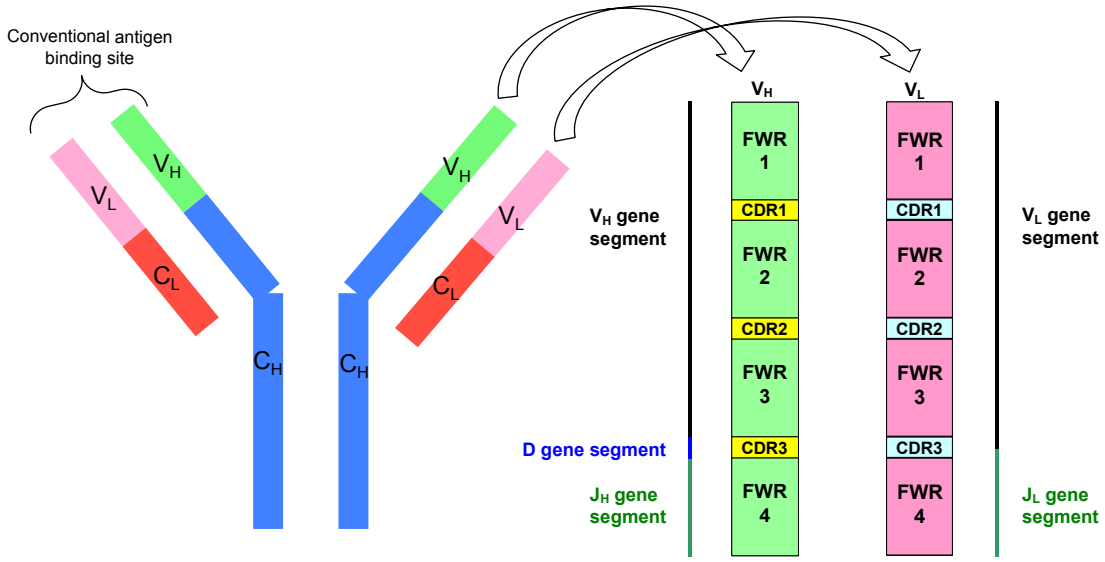


Figure 1.1: Schematic illustrating of antibody structure and arrangement of the V regions. The antibody structure comprising of two identical heavy (H) and two identical light (L) chains, each possessing variable heavy (V_H) and constant heavy (C_H) domains and variable light (V_L) and constant light (C_L) domains respectively. Both V_H and V_L are comprised of four framework regions (FWR) that provide the structural support for three complementarity determining regions (CDR). The V_H domain is encoded by a V_H gene segment, from the start of FWR1 through to the end of FWR3. CDR3 is encoded primarily by a D gene segment, but can include elements of the V_H-D and D-J_H junctions. A J_H segment encodes the final part of the V_H domain, FWR4. V_L domains are encoded by V_L and J_L gene segments from either the kappa or lambda gene loci. A V_L gene segment provides the template for FWR1 through to part of CDR3. A J_L gene segment encodes the remainder of CDR3 and FWR4.

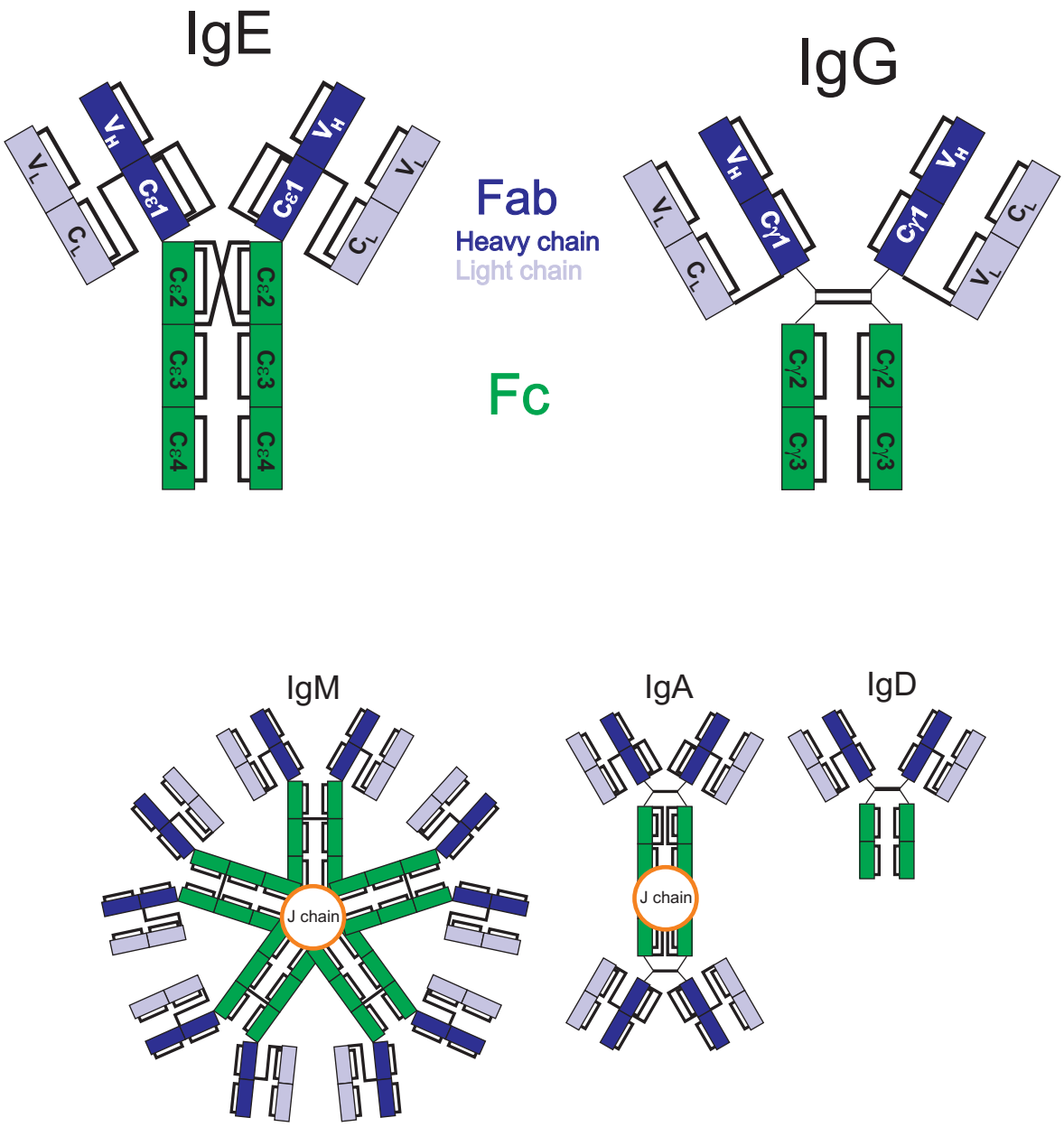


Figure 1.2: Schematic representation of the five main antibody isotypes, IgE, IgA, IgG, IgM and IgD. Fc portions, comprising isotype specific C_H domains 2, 3 (and 4) are shown in green and confer antibody effector functions. IgA, IgG and IgD Fc portion have two C_H domains and a hinge region, which is replaced by a third C_H domain in IgE and IgM. The Fab fragments are shown in purple – light purple for the light chain portion and dark purple for the heavy chain V_H and C_H1. Within each Fab fragment is the antigen binding site, comprised of hypervariable residues from the V_H and V_L domains. Antibody structure is stabilised by intra- and inter-domain disulphide bridges, illustrated by thick black lines. IgA and IgM are shown in their polymeric forms, with Fc portions linked by J chain polypeptides. Figure by Dr Rebecca Beavil.

In humans, the sequences of the framework regions of different light or heavy chains are relatively conserved, whilst antibodies with different specificities have different CDRs. Although it is the CDRs that vary from antibody to antibody, only a limited number of amino acid positions within the CDRs are directly involved in antigen binding. These positions within the CDRs are called specificity determining residues (SDRs). The variable domains of an antibody molecule are so termed due to the considerable variation in the amino acid sequence of this region observed throughout the expressed antibody repertoire. The genetic structures of the chromosomal loci that encode antibodies have evolved to enhance the available range of antibody specificities. At each of the three antibody gene loci, on chromosome 14 for the heavy chain, chromosome 2 for the kappa light chain and chromosome 22 for the lambda light chain, the isotype-specific constant domain exons are preceded upstream by a number of gene segments that recombine at random to generate the V_H, Vκ or Vλ domain. The V_L domains are encoded by a recombined V and Joining (J) gene segment, which joins the V_L to the appropriate C exon. In the V_H domains an additional Diversity (D) gene segment is recombined with the selected V and J gene segments to generate the complete V_H encoding DNA. Variability at the V(D)J joining site, or junctional diversity, is further enhanced by the deletion or addition of nucleotides during the joining process. The V(D)J junction encoded CDR3 loops, in particular the CDR3 of the heavy chain, constitute the most variable region of the whole antibody (Janeway et al, 2001).

The cell surface receptors (FcR) specific for the Fc domain are expressed on all cells of the immune system and play an important role in immune regulation, by linking the antibody-mediated immune responses with cellular effector functions. They are specific for each antibody isotype – FcαR for IgA, FcδR for IgD, FcεR for IgE, FcγR for IgG

and FcμR for IgM. In the case of FcγR, three distinct classes have been recognised: FcγRI, FcγRII, and FcγRIII, with different IgG binding affinities and IgG subclass specificities. The structural heterogeneity of FcγR is reflected in a wide range of biological activities, including clearance of antigen/antibody immune complexes, regulation of antibody production, enhancement of antigen presentation, antibody-dependent cell-mediated cytotoxicity or phagocytosis, degranulation, and activation of inflammatory cells. Many of these responses may be unique for selected cell types based on differences in tissue-specific expression of FcγRs (Janeway et al, 2001). The IgE receptors FcεRI (the high-affinity receptor) and FcεRII (the low-affinity receptor, CD23) participate in the afferent phase of the immune response to allergens. The allergens from the environment are internalised and processed by the Langerhans cells in epidermal tissues and transported as peptide-MHC class II antigen complexes to the local lymphoid tissues to amplify the immune response and reimplant memory of the allergen for future responses (Gould et al, 2003).

Each of the five classes of antibodies has a distinct structure, receptor specificity and function in the immune system (Janeway et al, 2001) (Table 1.1). Although there are differences in the actions of different antibody classes, all of them contribute to disable antigen in one way or the other. Functions of antibodies include:

1. Neutralisation – The interaction of an antibody with its cognate antigen blocks or neutralises certain bacterial toxins, and prevents the attachment of bacteria and viruses to the cells.

2. Enhancing opsonisation/phagocytosis – Antibodies bound to an antigen (via their variable region) can interact with their receptors on cells such as macrophages, neutrophils, basophils and mast cells (via their constant region) allowing them to identify a foreign antigen.

3. Complement activation – Antigen-antibody complexes initiate the classical pathway of the complement system and enhance phagocytosis of infected cells.

Isotype	Serum Conc. (mg/ml)	Serum half-life (days)	Main effector functions	Receptors
IgD	0.03	3	Binding to mast cells & basophils Neutralizing airway microbes	IgD-R
IgM	1.5	10	Classical pathway of complement activation; Neonatal immunity	FcμR
IgG1	9.0	21	Classical pathway of complement activation Fc receptor–dependent phagocytosis Neonatal immunity	FcγRI (CD64) FcγRIIA (CD32) FcγRIIB1 (CD32) FcγRIIB2 (CD32) FcγRIIIA (CD16a) FcγRIIIB (CD16b)
IgG2	3.0	20		
IgG3	1.0	7		
IgG4	0.5	21		
IgA1	3.0	6	Mucosal immunity: secreted into lumens of respiratory and gastrointestinal tracts Alternative pathway of complement activation	Polymeric IgR FcαRI (CD89) Fcα/μR
IgA2	0.5			
IgE	5×10^{-5}	2	High-affinity binding to mast cells and basophils (immediate hypersensitivity reactions)	FcεRI(high-affinity) FcεRI (CD23)

Table 1.1: Human serum antibody levels, their receptors and main function.

1.3 Conventional methods for antibody generation

Monoclonal antibodies include genetically engineered forms such as chimaeric, humanised or fully human antibodies (Janeway et al, 2001). A monoclonal antibody is an antibody produced by a single clone of B-lymphocytes or by a cell into which the light and heavy chain genes of a single antibody have been transfected. The chimaeric antibody comprises sequences derived from two different antibodies, which are typically derived from different species and usually represent human constant regions and murine variable regions. Typically variable segments of the genes from a mouse monoclonal antibody are joined to human constant segments by genetic engineering. A humanised antibody is an antibody that includes human framework regions and one or more CDRs from a non-human antibody. Typically, transferring CDRs from heavy and

light variable chains of a murine immunoglobulin into a human variable domain produces humanised monoclonal antibody (Jones et al, 1986; Riechmann et al, 1988; Verhoeyen et al, 1988). The use of antibody components derived from humanised monoclonal antibodies obviates potential problems associated with the immunogenicity of the murine portion. A fully human antibody is an antibody that includes human framework regions and all of the CDRs from a human immunoglobulin.

Different methods for antibody generation have been established. Methods for synthesising polypeptides and immunising a host animal to generate antibodies against the immunising polypeptides are well known. Typically, the host animal is inoculated intraperitoneally with an amount of immunogen and hybridomas are prepared by fusing lymphocytes from immunised animals with immortalised myeloma cells using the general somatic cell hybridisation technique (Kohler & Milstein, 1975). Hybridomas that produce suitable antibodies may be grown *in vitro* or *in vivo* and monoclonal antibodies are subsequently isolated from the culture media or body fluids, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel electrophoresis, dialysis, chromatography, and ultrafiltration. Undesired activity, if present, can be removed by running the preparation over adsorbents made of the immunogen attached to a solid phase and eluting or releasing the desired antibodies off the immunogen. If desired, the antibody of interest may be sequenced and the polynucleotide sequence may then be cloned into a vector for expression or propagation. The sequence encoding the antibody may be maintained in a vector in a host cell and the host cell can then be expanded and frozen for future use.

Another technique, Phage display, may be used to select and produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable domain gene repertoires

from unimmunised donors (McCafferty et al, 1990). Existing antibody phage display libraries may be panned in parallel against a large collection of synthetic polypeptides. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus antibody sequences selected using phage display from human libraries may include human CDR or variable region sequences conferring specific binding to antigens, which may be used to provide fully human antibodies with therapeutic potential.

Other methods for deriving heavy and light chain sequences from human B cell and plasma cell clones are typically performed using polymerase chain reaction (PCR) techniques (Kuppers, 2004; Tiller et al, 2008), providing fully human variable region sequences.

More than a century since the discovery of antibodies, these molecules have a diverse range of applications. One such application is their use as treatment modalities for a range of diseases, including a number of human malignancies.

1.4 Cancer Immunotherapy

Cancer Immunotherapy is the use of the immune system to reject cancer. The main premise is stimulating the patient's immune system to attack the malignant tumour cells that are responsible for the disease. This can be either through immunisation of the patient, in which case the patient's own immune system is trained to recognise tumour

cells as targets to be destroyed, or through the administration of therapeutic antibodies as drugs, in which case the patient's immune system is recruited to destroy tumour cells by the therapeutic antibodies. Since the immune system responds to the environmental factors it encounters on the basis of discrimination between self and non-self, many kinds of tumour cells that arise as a result of the onset of cancer are more or less tolerated by the patient's own immune system since the tumour cells are essentially the patient's own cells that are growing, dividing and spreading without proper regulatory control. In spite of this fact, however, many kinds of tumour cells display unusual antigens that are either inappropriate for the cell type and/or its environment, or are only normally present during the organism's development (e.g. fetal antigens). Other kinds of tumour cells display cell surface receptors that are rare or absent on the surface of healthy cells, and which are responsible for activating cellular signal transduction pathways that cause the unregulated growth and division of the tumour cell. Such antigens and receptors can be used as markers or targets for the diagnostic or treatment of cancer.

1.5 Melanoma

Melanoma is an immunogenic, highly aggressive and most lethal form of skin cancer arising from malignantly transformed melanocytes. It affects people of all ages and incidence rates of melanoma have been rising by 5% per year, faster than any other cancer in the UK (Karim-Kos et al, 2011). Currently, surgical excision remains the standard of care for the treatment of primary melanomas. Excision of thin tumours less than 1 mm in thickness can result in an over 95% five-year survival rate (Balch et al, 2009). Despite this however, 20% of primary melanomas will progress to metastatic disease for which the treatment options are limited and the prognosis poor with a

median survival of 8-18 months (Balch et al, 2009). Therefore, effective treatments are urgently needed for patients with this disease.

Significant progress has been made in the treatment of advanced stage disease, with the US Food and Drug Administration (FDA) approval of Vemurafenib (BRAF V600E kinase inhibitor), Pegylated IFN-α2β (modified IFN-α2β, with increased half life and enhanced therapeutic efficacy) and Ipilimumab (fully human mAb targeting the inhibitory molecule cytotoxic T-lymphocyte antigen 4) (Lacy et al, 2012). The recent approval of the monoclonal antibody Ipilimumab for the treatment of melanoma suggests that activating immune responses with antibodies may have therapeutic significance and has renewed interest in the field of antibody therapies for the treatment of challenging tumours such as melanoma (Culver et al, 2011; Kaehler et al, 2010; Natarajan et al, 2011). However, despite the partial success and promise of various immunotherapeutic strategies, including antibodies, there are presently no promising antibody therapies that directly target antigens on the surface of melanoma cells.

1.6 HMW-MAA as a suitable target for antibody immunotherapy

The High Molecular Weight Melanoma Associated Antigen (HMW-MAA), also known as Melanoma-Associated Chondroitin Sulfate Proteoglycan (MCSP), Human Melanoma Proteoglycan (HMP), Melanoma-Associated Proteoglycan Antigen (MPG), Melanoma Chondroitin Sulfate Proteoglycan (mel-CSPG) or CSPG4 (Natali et al., 1984), has been identified as an appropriate target for antibody immunotherapy. It is a human melanoma-associated integral membrane chondroitin sulfate proteoglycan that plays a role in stabilising cell-substratum interactions during early events of melanoma cell spreading on endothelial basement membranes. The antigen consists of two noncovalently associated glycopolypeptides with apparent molecular weight of 280K

and 440K. The HMW-MAA core protein is initially translated as a precursor with a molecular mass of 240K with asparagine N-linked oligosaccharides of the high mannose type. It is over-expressed by more than 80% of human malignant melanoma cells (Esko et al, 1988; Spiro et al, 1989), including cancer-initiating cells, but not normal melanocytes. Staining of various types of tumours with a HMW-MAA-specific monoclonal antibody has shown that its expression is not restricted to melanoma. For solid tumours, HMW-MAA is also expressed on tumours of neuroectodermal origin (astrocytomas, gliomas, and neuroblastomas), squamous cell carcinoma of the head and neck, basal breast cancer, mesothelioma, pancreatic carcinoma, some types of renal cell carcinoma, chordoma, chondrosarcoma and soft tissue sarcomas (Wang et al, 2010a; Wang et al, 2010b). Its expression on primary and metastatic lesions and limited heterogeneity across tumours, represents a highly suitable therapeutic target (Campoli et al). Furthermore, the expression in malignant lesions does not vary following treatment with chemotherapeutic agents (Ferrone et al, 1993).

The presence of the HMW-MAA in activated pericytes in tumour-associated angiogenic vasculature suggests a role in regulating and promoting tumour angiogenesis (Maciag et al, 2008). This could offer an additional advantage for antibody therapy, not only in targeting HMW-MAA-expressing tumour cells, but also in restricting angiogenesis and reducing tumour cell growth and migration. HMW-MAA enhances motility, migration and the metastatic capacity of melanoma cells by enhancing interactions with the extracellular matrix. It may also act as an auxiliary growth factor and has a role in melanoma cell proliferation (Yang et al, 2009; Yang et al, 2004). This gives the hope of preferential elimination of the most proliferative and metastatic HMW-MAA-expressing melanoma cells by specific antibodies.

1.7 Rationale for the choice of HMW-MAA-specific monoclonal antibody

Pre-clinical and clinical studies have investigated the efficacy of HMW-MAA-directed immunotherapy of melanoma. A murine monoclonal IgG antibody 225.28s raised against an epitope of the human HMW-MAA has been generated by immunising female BALB/c mice with human melanoma M21 cells (Imai et al, 1980). The original mouse clone was made in a hybridoma format and variable region sequences published (Neri et al, 1996). The antibody reacted strongly with most of the melanoma cells tested and the epitope recognised by the mAb 225.28s was proven to be located on the core protein of the HMW-MAA (Kantor et al, 1986; Wilson et al, 1981). It has been established that the antibody binds with high affinity and specificity to the epitope and its efficacy was tested in a number of *in vitro* and *in vivo* models (Ferrone & Kageshita, 1988; Hafner et al, 2005). Moreover, the mAb 225.28S was found to bind to an epitope distinct from those detected by other HMW-MAA specific antibodies and was found to exhibit univalent binding with no detectable endocytosis, suggesting that the mAb 225.28s remains on the membrane of melanoma cells (Temponi et al, 1992). These early *in vitro* studies also show that the murine 225.28s antibody weakly activates both complement and cell-mediated melanoma cell toxicity. The main mechanisms associated with reduced proliferation and neovascularisation, restriction of cell migration and metastasis have recently been reported in the context of triple-negative breast cancer cells which express the antigen (Wang et al, 2010a). Furthermore, the ability of murine 225.28s IgG antibody to suppress melanoma tumour growth has been demonstrated in a human xenograft grown *s.c.* in severe combined immunodeficiency (SCID) mice (Hafner et al, 2005), but the mechanisms of its function were not fully analysed and it was conceded by the authors that the restricted access of IgG antibodies to solid tissues was a likely explanation for the modest extent of tumour regressions observed. Furthermore, the murine antibody is expected to induce HAMA (human anti-murine antibody) responses

in patients, resulting in neutralisation of the antibody and rapid clearance from the circulation, thereby significantly reducing any efficacy against tumour cells. In addition, the murine Fc region is not expected to effectively recruit FcR-expressing human immune effector cells which may target and kill tumour cells by mechanisms such as cytotoxicity and/or phagocytosis.

Therefore, engineering HMW-MAA-specific antibodies with human constant regions that may trigger human Fc-expressing effector cell functions could help us evaluate novel immunotherapeutic tools for the treatment of solid cancers, and will enrich our understanding of therapeutically-relevant antibody-mediated mechanisms of action against cancer cells.

1.8 IgG in cancer immunotherapy

The use of monoclonal antibodies in cancer immunotherapy has become a successful and important treatment modality in the clinical management of malignancies over the past 15 years. Currently, 12 antibodies have been approved by the FDA for treatment of cancers, and a large number of antibodies are being tested in early- and late-stage clinical trials (Scott et al, 2012). However, nearly all antibodies developed in clinic today rely on only one of the nine human antibody classes (IgM, IgD, IgG1-4, IgA1, IgA2 and IgE), namely IgG1, the most abundant antibody class in the blood. The preference for IgG1 in cancer immunotherapy arose from the early work of Neuberger, showing that IgG1 was the most efficacious of nine different antibody classes in complement-mediated lymphoma cell killing by human peripheral blood mononuclear cells (PBMC) *in vitro* (Bruggemann et al, 1987). Subsequent clinical trials with antibodies recognising the B cell marker, CD20, supported the inference that IgG1 would be the subclass best suited for immunotherapy of patients with B cell

malignancies such as non-Hodgkin's lymphoma (Alduaij & Illidge, 2011). However, the human immune system naturally deploys the nine antibody classes to perform immune surveillance and to mediate destruction of pathogens in different anatomical compartments. Whilst IgG antibodies have been shown to be efficacious, it is known that they diffuse inefficiently into solid tissues, and bind with low affinity to their receptors on immune effector cells.

It is now known that complement-mediated tumour cell death is only one of several mechanisms by which antibodies may mediate tumour growth restriction (Weiner, 2007). Other known mechanisms include engaging immune effector molecules through their Fc regions to induce immune cell-mediated destruction of targeted cells by antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis (ADCP) (Figure 1.3). Antibodies can also act directly on tumour cells to inhibit growth signalling pathways, induce apoptosis, restrict proliferation and cell differentiation of tumour cells, or block tumour cell adhesion and migration. The poor tissue penetration of IgG antibodies and low affinity of IgGs for their receptors on immune cells may partly account for the weak immune responses observed and resulting poor performance of many IgG antibodies against solid tumours. Therefore, there is still a need for improved therapeutic antibodies, particularly for the treatment of neoplastic diseases such as skin cancer. In particular, there is a need for antibodies having improved effector functions compared to IgG antibodies, which may lead to an improved clinical outcome in the treatment of cancer, especially melanoma.

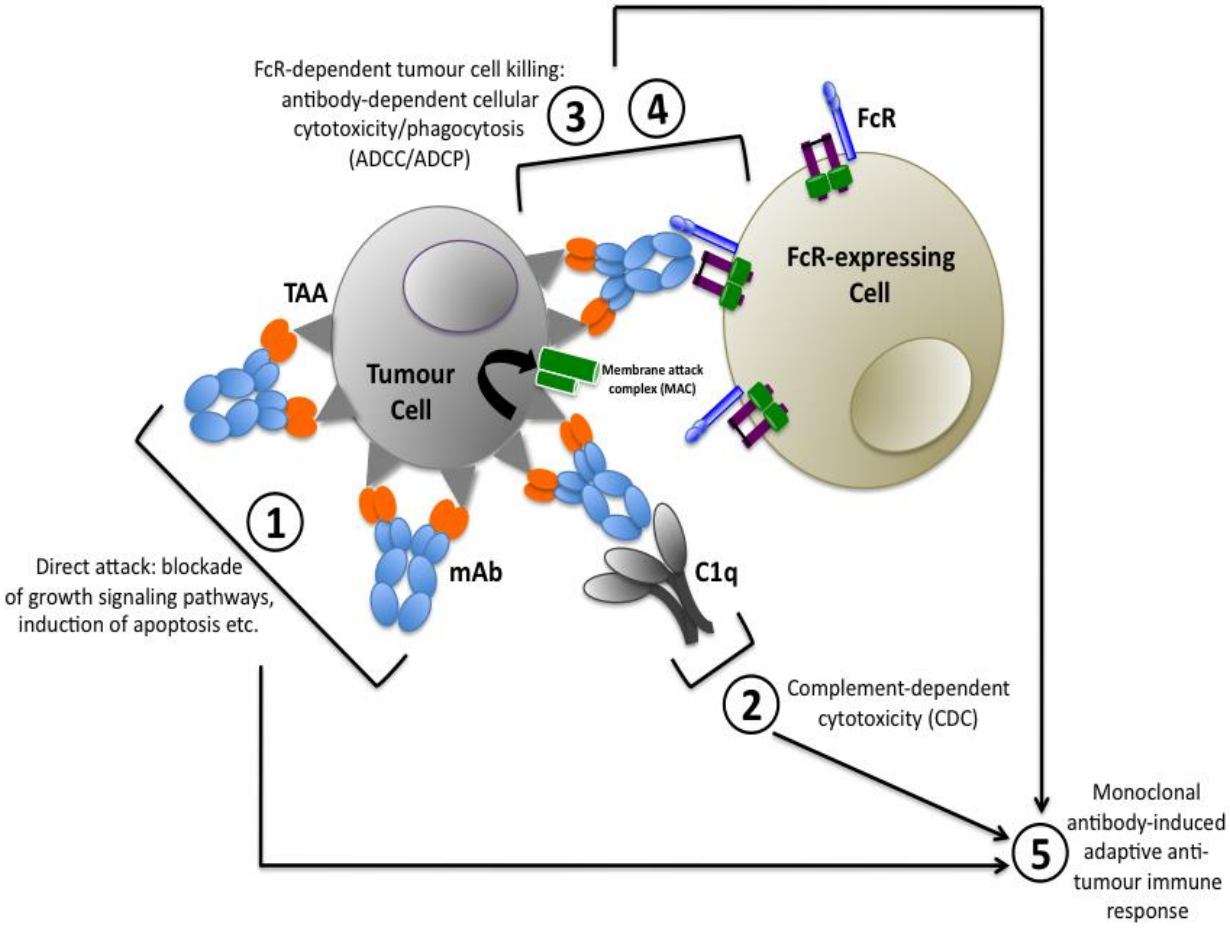


Figure 1.3. Schematic representation depicting the mechanisms of action of anti-tumour monoclonal antibodies. Antibodies, with specificity to TAAs, may operate through several mechanisms to kill cancer cells. These include 1 direct attack resulting in blockade of growth signalling pathways, restriction of proliferation and cell differentiation, or induction of apoptosis; 2 complement-dependent cytotoxicity (CDC); 3 antibody-dependent cellular cytotoxicity (ADCC); or 4 antibody-dependent cellular phagocytosis (ADCP). In favourable situations, these mechanisms may also lead to the induction of long-lasting adaptive anti-tumour immune responses 5. TAA = tumour associated antigen, mAb = monoclonal antibody, FcR = Fc receptor. Figure courtesy of Dr. Debra Josephs (KCL)

1.9 Rationale for using IgE for the treatment of solid tumours

IgE antibodies, known for their role in the allergic response and parasite protection, function through high-affinity Fc receptors, FcεRI and CD23, on a different spectrum of effector cells to IgG, and naturally reside in tissues where they exert immunological surveillance. These properties may translate to superior efficacy in targeting tissue-resident tumours such as melanoma. IgE is actively transported into tissues by the low affinity IgE receptor FcεRII (also known as CD23), a mechanism that is not available to IgGs (Gould et al, 2003), and retained locally by powerful IgE receptor-expressing resident cells. The affinity of IgE for its high-affinity receptor, FcεRI, (K_a=10^{11} M^{-1}) is 10^2-10^5 times higher than that of IgG1 for FcγRIII (the antibody receptor responsible for IgG1-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) of tumour cells), making it the only antibody strongly retained by effector cells in the absence of antigen (Gould & Sutton, 2008; Kinet, 1999; Ravetch & Kinet, 1991). The slow dissociation of the IgE- FcεRI complex and local retention of IgE in tissues may translate to lower effective therapeutic doses and/or reduced frequency of administration compared to IgG. The half-life of IgE in tissues has been shown to be of the order of two weeks, which is proportionately longer than that of IgG (2-3 days) (Gould et al, 2003; Hellman, 2007). Unlike IgG, IgE is subject to no inhibitory receptor (*cf*. FcγRIIb) (Clynes et al, 2000).

IgE antibodies are primarily known for their role in immediate hypersensitivity reactions – the basis of condition's such as allergic rhinitis and asthma. IgE antibodies against allergens remain permanently bound to IgE receptors on the surface of mast cells and Langerhans cells. The cross-linking of FcεRI on mast cells by allergen, leads to the release of histamine, leukotrienes, proteases and cytokines which increases circulation to the tissue and the influx and activation of inflammatory and potentially

cytotoxic cells (monocytes, eosinophils, basophils) at the site of allergen provocation. These activated cells may also migrate to local lymph nodes to stimulate T-cells, which in turn migrate to the tissue (Gould et al, 2003). In the context of their protective role in parasitic infections, IgE antibodies are known to trigger both ADCC, and antibody-dependent cell-mediated phagocytosis (ADCP) of parasites. Both IgE receptors (FcεRI and CD23) are up-regulated by IgE and IL-4 on effector cells *in situ*, and are known to participate in these mechanisms of action (Gould et al, 2003).

These properties of IgE antibodies may be redirected to enhance cytotoxicity and phagocytosis of tumour cells, as well as initiate IgE antibody-dependent antigen presentation by IgE receptor-bearing antigen-presenting cells such as dendritic cells, B cells and macrophages. Thus, passive and active immunity against solid tumours could act in conjunction in tissues such as skin, naturally populated by IgE effector cells. The strength of IgE-mediated immune responses in tissues, then, carries the expectation of increased potency as well as longevity of immune surveillance by IgE and effector cells against skin tumours.

The main reservation about clinical use of IgE has been a perceived risk of inducing Type I hypersensitivity that may, in some circumstances contribute to systemic anaphylaxis. The most important basic principle in Type I hypersensitivity is that only allergens which are able to target more than one IgE bound to FcεRI on effector cells will lead to productive crosslinking and mediator release (Larche et al, 2006). Therefore, Type I hypersensitivity may occur when IgE, attached to FcεRI on circulating basophils and lung/mucosal mast cells, is cross-linked by soluble multivalent antigens. To avoid this circumstance, a target antigen that is not shed in a multivalent form into the circulation must be chosen: indeed the HMW-MAA is monomeric in its

soluble form. In addition, the tolerability of a fully human anti-HER2/neu IgE antibody in a preliminary study conducted in Macaca fascicularis (cynomolgus) monkeys has been reported (Daniels et al, 2012).

Previously, we have reported the therapeutic efficacy of MOv18, an IgE antibody recognising the tumour-associated antigen Folate Receptor α (FRα) which is expressed in 70% of ovarian carcinomas and by a proportion of other cancers such as mesothelioma, breast and melanoma cancers. MOv18 IgE was more effective than the corresponding IgG1 in eliciting immune responses against cancer in two mouse xenograft models of ovarian carcinoma (Gould et al, 1999; Karagiannis et al, 2003). Mechanistically, MOv18 IgE induced monocytes to kill up to 70% of ovarian carcinoma cells; tumour killing was mediated by two mechanisms: ADCC and ADCP of tumour cells, both known IgE mechanisms of action in protection from parasitic infections. The antibody did not exert any direct effects on tumour cell viability or proliferation (Bracher et al, 2007; Karagiannis et al, 2008b; Karagiannis et al, 2007). Furthermore, a specifically engineered trastuzumab IgE was as effective as trastuzumab IgG1 at directly mediating tumour cell growth arrest in cellular viability (MTT) assays *in vitro* (Karagiannis et al, 2009). In addition, both trastuzumab IgG1 and IgE antibodies activated monocytic cells to kill tumour cells, but operated by different mechanisms: trastuzumab IgG1 functioned by ADCP, whereas trastuzumab IgE, unlike MOv18 IgE in ovarian carcinoma, functioned by inducing moderate levels of ADCC only.

Based on all these properties of the IgE antibodies and their activity in our ovarian carcinoma models, we suggest that they may demonstrate both superior tissue bioavailability and higher receptor affinity. This may result in improved efficacy of immunotherapy for solid tumours by the use of IgE antibodies in place of the currently

conventional IgG. We therefore wished to conduct a direct comparison, based on engineered chimaeric 225.28s antibodies of different classes, IgG1 and IgE, and examine their potential efficacy in the treatment of melanoma.

1.10 Project aims

Guy's & St Thomas' NHS Foundation Trust (GSTFT) and King's College London (KCL) are strategic and academic partners of one of five National Institute for Health Research (NIHR) comprehensive Biomedical Research Centres (BRC) under the Department of Health's strategy for R&D. The aim of the GSTFT/KCL NIHR Biomedical Research Centre is to create a unique Centre that enables a pipeline of excellent basic biomedical research to deliver real change in the management of the patients. The BRC focuses on seven research themes encompassing Asthma & Allergy, Atherosclerosis, Cutaneous Medicine, Cancer, Immunity and Infection, Oral Health and Transplantation and has cross cutting disciplines encompassing Genetics, Paediatrics, Imaging Sciences, Stem Cell Research, Cell and Molecular Biophysics, Age-related diseases and Health & Social Care Research. The "Discovery to Phase I trials of Novel Therapeutic Antibodies for Cancer" program within the Biomedical Research Centre (BRC) led by Dr. James Spicer, Dr. Sophia Karagiannis, Prof. Philip Blower, Prof. Hannah Gould & Dr. Andrew Beavil aims to:

1) isolate patient derived melanoma antigen-specific antibody variable gene sequences
2) re-format these into recombinant monoclonal antibodies
3) test the function and efficacy of newly discovered recombinant monoclonal antibodies *in vitro* and *in vivo*

The BRC engaged three PhD students to deliver its program aims, one for each aim. My contribution to the program was to deliver aim 2, but inevitably I was closely involved

in aim 1 and 3. My main objectives include:

a) implementation and optimisation of our existing transient antibody expression method to enhance transfection efficiency, increase production and further support the characterisation of an existing melanoma antigen-specific antibody (Chapter 3)

b) improvement of antibody cloning and expression by constructing a dual antibody expression cassette using an alternative to conventional restriction enzyme- and ligase-dependent cloning methods, allowing time-efficient generation of stable cell lines for production of fully functional recombinant antibodies in large quantities (Chapter 4)

c) Production and characterisation of melanoma antigen-specific IgG1 and IgE, followed by parallel comparative functional studies between the two isotypes using validated *in vitro* and *in vivo* assays (Chapter 5)

d) Supporting the antibody discovery program by re-formatting the isolated patient derived melanoma antigen-specific variable gene sequences as whole antibodies, followed by their characterisation and validation *in vitro* (Chapter 6)

2 MATERIAL AND METHODS

2.1 General reagents and solutions

All materials and reagents used in this thesis are shown in the table below:

Product name	Supplier	Catalogue number
0.2ml PCR tube, flat cap, natural	Elkay Laboratory Products (UK) Ltd	THER-02NF
1kb DNA Ladder	New England Biolabs	N3232S
2-isopropanol	Fluka	59310
2-log DNA ladder (0.1 - 10kb)	NEB	N3200S
2-Mercaptoethanol 50mM 20ml	Gibco	31350-010
2.0ml Cryovial Internally Threaded Self-Standing Sterile	Alpha Laboratories Limited	LW3334
Acrylamide Solution, Pre-made, 30% (w/v) Ratio 37.5	Severn Biotech Ltd	20-2100-05
Agarose	Bioline	BIO41025
Agarose, Molecular Grade	Bioline Limited	BIO-41026
Amicon Ultra -15 Ultra cel- 30k	Millipore	UFC903008
Ammonium Persulfate	Sigma-Aldrich	A3678
Antarctic Phosphatase 5000U/ml	NEB	M0289S
BsiW I	New England Biolabs	R0553S
BssH II	New England Biolabs	R0199S
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2650
DNA Sequencing Vouchers	Source BioScience AUTOGEN	GSL1501
Dual Mini Gel Cast Set	Genetic Research Instrumentation Ltd	2393010
Dulbecco's Modified Eagle Medium (D-MEM) (1X), liquid (high glucose)	Invitrogen Life Technologies	11960085
Dulbecco's Phosphate Buffered Saline (D-PBS) (1X), liquid	Invitrogen Life Technologies	14190169
EcoR1 10,000U	NEB	R0101S
EcoRI-HF	New England Biolabs	R3101S
Ethanol	Sigma-Aldrich	45-986-6
Ethidium Bromide 10mg/ml	Sigma-Aldrich	E1510-10ML
F-12 Nutrient Mixture (Ham), liquid	Invitrogen Life Technologies	21765029
Flp-In™ Complete System	Invitrogen Life Technologies	K6010–01
Flp-In™-293 Cell Line	Invitrogen Life Technologies	R75007
Flp-In™-CHO	Invitrogen Life Technologies	R758–07
Foetal Bovine Serum (FBS)	Invitrogen Life	10106169

	Technologies	
Geneticin® Selective Antibiotic, liquid	Invitrogen Life Technologies	10131027
Glycerol	Sigma-Aldrich	G-6279
Glycine	Sigma-Aldrich	G-7403
HEK-293E	ATCC	CRL-1537
Hind III	New England Biolabs	R0104S
HindIII 10,000U	NEB	R0104S
HiSpeed ® Plasmid Maxi Kit	QIAGEN	12663
HiTrap™ NHS-activated HP Column	GE Healthcare	17-0717-01
Hydrochloric acid ACS reagent, 37%	Sigma	258148-2.5L
Hygromycin B in PBS 50mg/ml	Invitrogen	10687-010
KpnI 3000U	NEB	R0142S
LB Agar, powder (Lennox L Agar)	Invitrogen Life Technologies	22700025
LB Broth Base, powder (Lennox L Broth Base)®	Invitrogen Life Technologies	12780052
Marvel, dried skimmed milk	Premier International Foods	
MEM 10 x 500ml	Invitrogen	31095-052
MEM Non Essential Amino Acids (100X), liquid	Invitrogen	11140-035
Methanol	Fisher Scientific	M/3950/PK4
Microcentrifuge tube with lip seal screwcap and loop polypropylene tube polyethylene cap Fisherbrand 1.5mL	Fisher Scientific Ltd	FB74345
N,N,N′,N′-Tetramethylethylenediamine for molecular biology, ≥99% (GC)	Sigma	T7024-25ML
Nar I	New England Biolabs	R0191S
NheI-HF	New England Biolabs	R3131S
NruI 1000U	NEB	R0192S
Nunc-Immuno™ Plates 96-well plate, Maxisorp	Sigma	M9410-1CS
o-Phenylenediamine dihydrochloride 5mg tablet	Sigma-Aldrich	P6912-100Tab
o-Phenylenediamine Dihydrochloride tablet, 5 mg substrate per tablet	Sigma	P6912-100TAB
OptiMAb® Monoclonal Antibody Production Enhancer (100X), liquid	Invitrogen	11910-031
Penicillin-Streptomycin-Glutamine (100X), liquid 100ml	Invitrogen Life Technologies	10378-016
Penicillin-Streptomycin, liquid	Invitrogen Life Technologies	15140122
Pens, lab marker, extra fine tip, black,	VWR International	811-0060
Phusion Flash High-Fidelity PCR Master Mix	New England Biolabs	F-548S
Plasmid Maxi Kit (25), 25 QIAGEN-tip 500, Reagents, Buffers	QIAGEN	12163
Polyclonal Goat Anti-Human IgE-Peroxidase, Specific for Epsilon-Chains	Sigma	A9667
Polyclonal Rabbit Anti-Human IgE,	Dako	A0094

Specific for Epsilon-Chains		
Quick Ligation Kit	New England Biolabs	M2200S
QuikChange Lightning Site-Directed Mutagenesis Kit	Stratagene	210518
Sac II	New England Biolabs	R0157S
SDS Solution, 20%, 1000ml	Severn Biotech Ltd	20-4002-10
SEAKEM GTG AGAROSE	VWR International	733-1541
SeeBlue® Plus2 Pre-Stained Standard	Invitrogen Life Technologies	LC5925
Sfi I	New England Biolabs	R0123S
Sodium Azide	Sigma-Aldrich	S-2002
Sodium chloride	Sigma-Aldrich	S-9625
Sodium Hydrogen Carbonate	BDH Chemicals	30151
Spectrafuge™ Mini Centrifuge	Sigma	S7816GB-1EA
Stable Peroxide Substrate Buffer (10X)	Fisher Scientific Ltd	34062
Tissue culture flask TripleFlasks polystyrene Nunclon	Fisher Scientific Ltd	TKT-130-030V
Trypan blue solution, 0.4%	Sigma-Aldrich	T8154
TrypLE™ Express Stable Trypsin Replacement Enzyme without Phenol Red	Invitrogen Life Technologies	12604021
Tween20 (Polyoxyethylene (20) sorbitan monolaurate	Sigma-Aldrich	P-5927
Vacuum filter unit sterile Millipore 250ml funnel 250ml receiver	Fisher Scientific Ltd	FDR-120-050L
Wide Mini-Sub Cell GT System	BIO-RAD laboratories europe ltd	170-4468
Wizard Plus SV Minipreps DNA Purification System	Promega	A1330
Wizard SV Gel and PCR Clean-Up System	Promega	A9281
XbaI 5000U	NEB	R0145S
XhoI 5000U	NEB	R0146S
XL1-Blue Competent Cells	Stratagene	200249
XL10-Gold Ultracompetent cells	Stratagene	200314
XL2-Blue Ultracompetent Cells	Stratagene	200150

Table 2.1.1: Supplier details for general reagents and materials

All solutions used in this thesis are shown in the table below:

Solution	Constituents
0.2M Glycine	0.2M Glycine , pH 2.5, 0.1% NaN3
1 M Tris	121.14 g Trizma base, MilliQ water up to 1L, pH 8.6, 0.1% NaN3
10% APS	APS dissolved 10% w/v solution in MilliQ water
10X SDS-PAGE run buffer	30 g Trizma base, 144 g Glycine, 100 ml 10% SDS, MilliQ water up to 1L
5xSDS-Sample Buffer	3.125 ml 1 M Tris (pH 6.8), 5ml glycerol, 0.25 g SDS and 1 ml 2-mercaptoethanol (prior to analysis) to 10ml MilliQ water
Coomassie blue destain	5% v/v methanol, 7.5% v/v acetic acid in MilliQ water
Coomassie blue stain	0.05% coomassie blue powder in 45% v/v Methanol, 45% v/v MilliQ water and 9% v/v acetic acid
ELISA Blocking Buffer	2% milk powder (Marvel) in 1xPBS
ELISA Carbonate Buffer	1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, up to 1000 ml MilliQ water, pH 9.6
ELISA Wash Buffer	1xPBS-Tween20 (0.05%)
FACS Buffer	1xPBS supplemented with 5% Normal Goat Serum and kept at 4ºC
HPLC buffer	0.5 M Tris-HCl, 250 mM NaCl, 0.05% Sodium azide, pH adjust to 7.2, filtered
Lower Tris	1.5M Tris, 0.4% SDS, pH8.8
OPD Buffer	one 5 mg tablet of OPD in the 10 ml of Stable Peroxide Substrate Buffer
Stable Peroxide Substrate Buffer	1ml Stable Peroxide Substrate Buffer (10x) in 9ml MilliQ water
Upper Tris	0.5M Tris, 0.4% SDS, pH 6.8

Table 2.1.2: Solution reagents and their constituents

2.2 PCR and Sequencing Primers

Primers were obtained using the Custom DNA Oligo service from Sigma. The sequences of each primer used in this thesis are listed in the table below:

Primer	5' – 3' Sequence
MCS2_F	tgtacagctagctggccagacatgataagatacattgatg
MCS2_R	accggttgctttgaattagcggtggttttcacaacaccta
HC_F	gctaattcaaagcaaccggtatggactggacctggaggat
HC_R	tctggccagctagctgtacatcatttaccgggatttacag
pVitro1F	ttttgagcggagctaattctcggg
pVitro1R	aaaaaacctcccacacctcc
MCS1_F	ggatcccgtacgcctaggagcaggtttccccaatgacaca

MCS1_R	tccggattgctttgaattagcggtggctttcacaacacct
Linear_Kfwd	cgtacggtggcggcgccatctgtcttcatcttcccgccat
Linear_Krev	accgcggctagctggaacccagagcagcagaaacccaatg
Linear_Hfwd	gctagcacacagagcccatccgtcttccccttgacccgct
Linear_Hrev	ggagtgcgcgcctgtggcggccgccaccaagaagaggatc
MAAK_Fwd	gggttccagctagccgcggtgacatcgagctgacccagag
MAAK_Rev	aagacagatggcgccgccaccgtacgcttgatttccagct
MAAH_Fwd	ccgccacaggcgcgcactcccaagtcaaactgcagcagag
MAAH_Rev	gatgggctctgtgtgctagcgctgctgacagtcacggtgg
pAn_Fwd	tgtacagctagctggccagacatgataagatacattgatg
VH_Rev	gctgctgacagtcacggtggtgccctggccccagtggtcg
Cg4_Fwd	ccaccgtgactgtcagcagcgctagcaccaagggcccatc
Cg4_Rev	ccctgtctctgggtaaatgatgtacagctagctggccaga
pTT3-F	ccaaaaacgaggaggatttgatat
pTT3-R	tctccgagggatctcgaccaaatga
Gam1_Fwd	ccaccgtgactgtcagcagcgctagcaccaagggcccatc
Gam1_Rev	tctggccagctagctgtacatcatttacccggagacaggg
GAPDH_Fwd	atttggtcgtattgggcgcctggtc
GAPDH_Rev	tcatacttctcattgttcacacccatg
LinearG1_Fwd	gctagcaccaagggcccatcggtcttccccctggcaccct
M394H_Fwd	cacaggcgcgcactccgaggtgcagctggtggagtctggg
M394H_Rev	ggcccttggtgctagctgaagagacggtgaccacggttcc
M394K_Fwd	tccagctagccgcggtgatgttgtgatgactcagtctccactctccctgc
M394K_Rev	gcgccgccaccgtacgtttgatctccaccttggtccctcc
M80-F2H_Fwd	cacaggcgcgcactccgaggtgcagctggtggagtctggg
M80-F2H_Rev	ggcccttggtgctagctggggagacggtgaccagggttcc
M80-F2K_Fwd	tccagctagccgcggtgacatccagatgacccagtctcca
M80-F2K_Rev	gcgccgccaccgtacgtttgatttccaccttggtcccttg
M80-F4H_Fwd	cacaggcgcgcactcccaggtgcagctgcaggagtcgggc
M80-F4H_Rev	ggcccttggtgctagctgaggagacggagaccagggttcc
M80-F4K_Fwd	tccagctagccgcggtgacatccagatgacccagtctcca
M80-F4K_Rev	gcgccgccaccgtacgtttgatctgcagcttggtcccctg

Table 2.2: PCR and sequencing primers

2.3 LB Culture Medium

LB culture medium was made by dissolving LB powder (Invitrogen) in MilliQ water at a ratio of 20g LB per litre of water and the solution was autoclaved. The medium was kept at room temperature. Ampicillin (Sigma-Aldrich), was added at 100μg/ml, prior to culture inoculation.

2.4 LB Agar Culture Plates

To make LB agar culture plates, LB agar powder (Invitrogen) was added to MilliQ water at a ratio of 32g powder per litre of water and autoclaved. The molten LB agar was cooled to approximately 50°C before addition of ampicillin (Sigma-Aldrich) at 100μg/ml, and poured into 90mm diameter Petri dishes (20-25ml per dish) to set. Plates were stored at 4°C until used.

2.5 SOB Medium

SOB Medium required for SOC Medium preparation, was prepared by adding 20.0 g of tryptone (Sigma), 5.0 g of yeast extract (Fisher Scientific Ltd), 0.5 g of NaCl (Sigma), MilliQ water to a final volume of 1 liter and autoclaved. Then 10 ml of filter-sterilized 1 M $MgCl_2$ (Sigma) and 10 ml of filter-sterilized 1 M $MgSO_4$ (Sigma) were added. SOB Medium was kept at RT.

2.6 SOC Medium

Prepared by adding 2 ml of filter-sterilized 20% (w/v) glucose (Sigma) or 1 ml of filter-sterilized 2 M glucose and SOB medium (autoclaved) to a final volume of 100 ml. Kept at RT.

2.7 Transformation

General transformations were performed using XL1-Blue Competent Cells (Stratagene). Ligation products were transformed using XL2-Blue Ultracompetent Cells (Stratagene). When using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene), the DNA was transformed into XL10-Gold Ultracompetent cells. All transformations were done with the protocols recommended by Stratagene, using SOC (described above) for

XL1-Blue Competent Cells and LB (described above) for XL2-Blue and XL10-Gold Ultracompetent cells as the medium for outgrowth following the heat pulse. The transformation mixture was plated onto LB agar Petri dishes with 100μg/ml ampicillin.

2.8 Overnight Bacterial Cultures

Single colonies selected for growth LB agar Petri dishes were transferred by pipette tip into 4ml LB medium containing 100μg/ml ampicillin. Cultures were incubated overnight at 37°C with shaking at 250rpm.

2.9 Long term storage of bacterial strain

It is lab policy to prepare a frozen stock of newly created strains as soon as possible. 1 ml of the 4mls Overnight Bacterial Culture is used for long term storage of bacterial strain and the rest 3ml for mini-preparation of Plasmid DNA. 150 μl sterile 100% glycerol (Sigma) and 850 μl of the bacterial culture (frozen stock is 15% glycerol) were added to a sterile, labelled cryo-vial. This was mixed well and stored at -80°C. A miniprep was then performed for DNA purification.

2.10 Mini-preparation of Plasmid DNA from Overnight Cultures

Plasmid DNA was purified from 3ml overnight cultures using a Wizard Plus SV Minipreps DNA Purification System (Promega), following the kit's micro-centrifugation protocol. The concentration and purity of eluted DNA was measured with NanoDrop ND-1000 spectrophotometer at 260 nm and 280 nm wavelengths. The 260:280 ratios of the DNA preparations were typically between 1.80 – 1.93. The DNA was stored at -20°C. When DNA was needed for large-scale transfection a maxi-preparation of Plasmid DNA was used.

2.11 Maxi-preparation of Plasmid DNA from Overnight Cultures

Plasmid maxi preparation was done using Plasmid Maxi Kit (QIAGEN). As a starter culture, 1 µl from overnight culture was diluted in 5ml LB medium containing 100µg/ml ampicillin and incubated for approx. 8 h at 37°C with vigorous shaking (approx. 250 rpm). The culture was diluted 1/500 and inoculated in 500 ml LB medium containing 100µg/ml ampicillin. It was grown at 37°C for 12–16 h with vigorous shaking (approx. 2500 rpm). The DNA was then purified following the kit's protocol.

2.12 Restriction Digests

Once plasmid DNA has been purified from *E. coli* a diagnostic restriction digest was used for verifying the correctness of the DNA. All restriction enzymes used in this report were purchased from New England Biolabs. The digestion reactions were performed strictly following each restriction enzyme's recommended buffer and conditions. The restriction enzyme cutting site/sites on the template DNA and the expected band/bands sizes after the digest were estimated using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2). This tool takes a DNA sequence and finds the large, non-overlapping open reading frames using the *E.coli* genetic code and the sites for all commercially available restriction enzymes that cut the sequence. When double restriction digest was required (as common timesaving procedure), Double Digest Finder (http://www.neb.com/nebecomm/DoubleDigestCalculator.asp) was used, which selects reaction conditions amenable to any two NEB restriction enzymes. When performing a diagnostic restriction digest, 4 units of enzymes were sufficient to digest 500ng of DNA. The digestion of DNA for subsequent cloning required 10 units of enzyme for 2 µg of template DNA. Products of restriction digestions were separated by gel electrophoresis.

2.13 Agarose Gel Electrophoresis

DNA resulting from PCR and restriction digestions was separated by electrophoresis on 1% agarose gels. 1.5g agarose (Bioline) were dissolved in 150ml 1 x TBE by heating. The molten gel was allowed to cool before addition of 0.5 μg/ml ethidium bromide (Sigma-Aldrich) and poured into a gel tray. Once set, the gel was placed in an electrophoresis tank of 1 x TBE and the gel comb removed. Samples pre-mixed with Gel Loading Dye, Blue (6X) (New England Biolabs) were applied to wells, alongside a molecular weight marker, and run through the gel at 130V for approximately 45 minutes. DNA bands were visualised by exposure to UV light and photographed using a Syngene Gene Genius Bioimaging System (Cambridge, UK). However, when DNA resulting from PCR or Restriction Digestion was meant to be used for a ligation reaction, SeaKem® GTG® Agarose (VWR International) was used for DNA separation.

2.14 DNA Molecular Weight Markers

1 kb DNA ladder (New England Biolabs) and 100bp DNA ladder (New England Biolabs) were used to estimate the size of DNA fragments separated by gel electrophoresis (Figure 2.1). As each DNA fragment of the ladders were of known concentration, the markers could also be used to estimate the quantity of DNA in sample bands by intensity comparisons. Prior to first use these ladders were mixed with MilliQ water and an appropriate volume of Gel Loading Dye, Blue (6X) (New England Biolabs) as recommended.

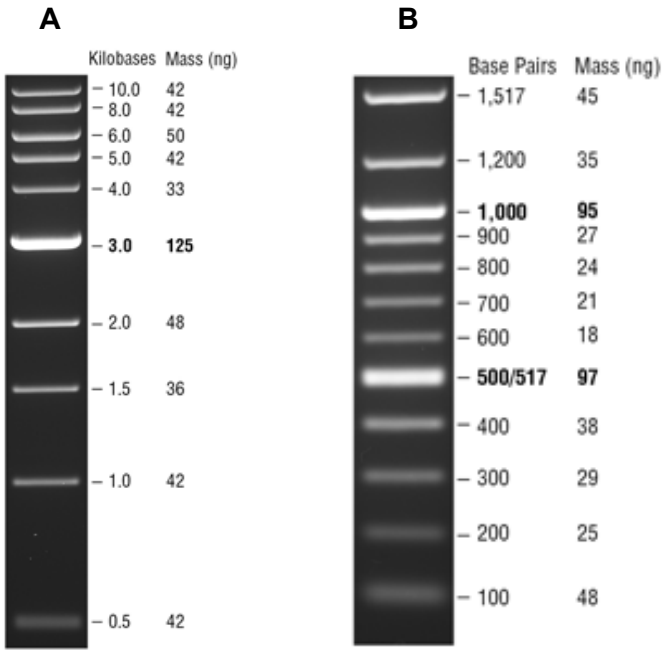


Figure 2.1: DNA ladders. Ladders visualized by ethidium bromide staining on a 0.8% TAE agarose gel. Mass values are for 0.5 µg/lane. a) 1 kb DNA Ladder; b) 100bp DNA Ladder. Images from www.neb.com .

2.15 DNA Sequencing Reactions

Once the diagnostic restriction digest had shown the correct bands, the DNA was sequenced using specific forward and/or reverse primer/s. 5µl plasmid DNA at 100ng/µl concentration and 5 µl primer at 3.2pmol/µl were used. Sequencing reactions were forwarded to the Geneservice (Source BioScience, Cambridge, UK) for processing on an Applied Biosystems 3730 DNA Analyzer. The data was emailed back to me and analysed on MacVector (V12.6)

2.16 PCR

Phusion™ Flash High-Fidelity PCR Master Mix (New England Biolabs) was used for general PCR procedures. The unique composition of Phusion Flash PCR Master Mix enables the use of extremely short PCR protocols (15 s/1 kb) with both low and high complexity DNA templates. The Master Mix contains all reagents required for PCR

except for the DNA template and primers. 20µl reactions were set up in which the primers were each used at 0.5µM. Reactions were initially denatured at 98°C for 2 minutes, followed by 30 cycles of 98°C denaturation for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 1 minute, then a hold at 4°C. PCR products were analysed by gel electrophoresis.

2.17 Colony PCR

Transformant colonies were analysed by PCR to ensure they had correctly taken up the plasmid DNA. This is very quick strategy and especially useful after a ligation reaction, giving the opportunity to quick-check the ligated insert and its orientation (when single restriction site has been used). It involves the use of a forward primer specific to the insert and a reverse primer with specificity to a portion of the vector, then adding a small amount of the selected single colony to act as a template. The following steps in this method are the same as for general PCR, except an initial 5 minutes denaturation.

2.18 DNA Gel Extraction and PCR Clean-Up

Restriction digest products of the appropriate size, used for subsequent cloning, were visualized by a long-wavelength UV lamp (as normal one would damage the DNA) and excised from SeaKem agarose gels using a razor blade. The masses of the gel slices were determined and the DNA purified using a Wizard® SV Gel and PCR Clean-Up System (Promega), following the kit's centrifugation protocol. The concentration of eluted DNA was measured with a NanoDrop ND-1000 Spectrophotometer and stored at -20°C.

2.19 Ligation reactions

All ligations were predominately carried out with inserts in excess to vectors. Typically, ligations were performed using 3:1 insert:vector ratio as calculated by

$$\frac{\text{ng of vector x kb size of insert}}{} \times \text{Molar ratio of insert/vector}$$

utilising the Quick Ligation Kit (New England Biolabs) according to manufacturer's recommendations. DNA extractions on transformed clones were typically carried out with a Wizard Plus SV Minipreps DNA Purification System (Promega). Positive clones were determined by diagnostic restriction digest followed by sequencing.

2.20 Cell lines

All cells were maintained in a 5% CO_2 humidified incubator at 37^{o}C.

HEK293E – Human Embryonic Kidney (HEK) 293, expressing the Epstein-Barr Virus nuclear antigen-1 (EBNA-1) (Durocher et al., 2002), were cultured in DMEM, 10% FBS, 1% Penicillin-Streptomycin-Glutamine and 250 μg/ml G418 to maintain selection of transfected EBNA-1 plasmid, and kept between 1 – 9 x 10^5 cells/ml.

293-F - Human Embryonic Kidney (HEK) 293-F Suspension FreeStyle™ cells (Life Technologies) were cultured in FreeStyle™ 293 Expression Medium.

RBL-SX38 – Cells of the Rat Basophilic Leukaemia (RBL) cell line, expressing both human and rat FcεRI (Dibbern et al., 2003), were cultured with MEM, 10% FBS, 1% Penicillin-Streptomycin-Glutamine and 1 mg/ml G418 and kept between 1 – 8 x 10^5 cells /ml.

U-937 - This non-adherent human monocyte-like cell line was also kindly provided by Professor J.P Kinet (Harvard University, Boston, MA, USA). Cells were maintained in RPMI 1640 media with standard additives at a density between 2 x 10^5 to 2 x 10^6 cells/mL. Where U937 cells were cultured in IL-4, this involved supplementation of the standard culture media with 320 U/mL recombinant human IL-4, four days prior to experiments.

A375 – Human Melanoma cell line, naturally expressing High Molecular Weight Melanoma Associated Antigen (HMW-MAA) (CRL-1619, ATCC, Manassas, VA) was grown in Dulbecco's Modified Eagles Media (DMEM) supplemented with10% FCS, 2 mM L-glutamine, penicillin (5000 U/mL) and streptomycin (100 µg/mL).

2.21 Thawing Adherent Cells

The following protocol has been designed to thaw adherent cells to initiate cell culture. All cell lines are supplied in vials containing 5 x 10^6 cells in 1 ml of Freezing Medium. The vial of cells was taken out from the liquid nitrogen and thawed quickly in a 37°C water bath. Just before the cells are completely thawed, the outside of the vial was decontaminated with 70% ethanol, and transferred to a 15 ml centrifuge tube containing 9 ml of culture medium. The cells were centrifuged for 5 minutes at 1000 rpm in Sorval RT 5000D centrifuge, fitted with the H1000B rotor. The medium was poured out and cells resuspended with 15 ml of fresh medium. They were transferred to a T-75 flask and incubated overnight in a humidified, 37°C, 5% CO_2 incubator. The next day selection agent was added to the flask to maintain selection of transfected plasmid (where required) at previously determined concentration. Cells were checked daily until 80-90% confluent and then passaged.

2.22 Passaging Adherent Cells

When cells were ~80-90% confluent, the medium was removed from the flask and the cells washed once with 10 ml PBS to remove excess medium and serum (as serum contains inhibitors of trypsin). A minimal amount of TrypLE™ Trypsin (Invitrogen) was used to coat the flask surface and incubated for 5 minutes at 37°C. The cells were observed under a microscope and it was confirmed that most of them had detached. If cells were not detached, longer incubation was needed. 10 ml of fresh medium was added to stop trypsinization. The solution was pipetted up and down to break up clumps of cells and 1 ml (to split them at 1/10) was transferred to a new 75 cm^2 flask and 14 ml fresh medium was added (with selection agent where required). Cells were incubated in a humidified, 37°C, 5% CO_2 incubator until 80-90% confluent and then passaged again.

2.23 Freezing Cells

Before starting, freezing medium (90% FCS, 10% DMSO) was prepared and kept on ice. Cell were detached from the flask (see Passaging Adherent Cells) and 10 ml of fresh medium was added. They were mixed, counted with haemocytometer (see Determination of Cell Numbers) and centrifuged for 5 minutes at 1000 rpm. The cells were then resuspended at a density of at least 5 $x10^6$ cells/ml in chilled freezing medium and 1 ml of the cell suspension was aliquoted into each cryovial. The cryovials were left in freezing box at -80°C. For ideal cryopreservation, the freezing rate should be a decrease of 1°C per minute. The next day the cryovials were transferred to liquid nitrogen for long-term storage.

2.24 Determination of Cell Numbers

Cells were diluted five times to a volume of 20µl in 0.4% Trypan Blue (Sigma-Aldrich). The total volume was loaded onto a Neuberger Improved haemocytometer and the dead cells, identified by their blue colour due to uptake of Trypan Blue, were excluded from the count. The recorded cell number was then multiplied by $5x10^4$ to give the number of live cells per ml.

2.25 Killing curve

Killing curves on HEK293E and HEK293F cells were performed to optimize selection of stable transfectants using the following concentrations of Hygromycin: 0 µg/ml, 50 µg/ml 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml. The cells were seeded at low densities, targeting 50% confluence (typically 5 x 10^5/ml), in 24 well plates in culture media. Varying concentrations of Hygromycin were added after overnight incubation for the cells attachment. After 10 days, cell viability was assessed visually, using the dye-exclusion method with trypan blue. Dead (blue) and viable (transparent) cells were then quantified using the haemocytometer. The lowest concentrations of the mammalian selection drug that had < 10% viable cells were determined as the minimal killing concentration (MKC) and used subsequently.

2.26 Transfection

All transfections in this report were performed using Polyethylenimine (Boussif et al., 1995) as transfection reagent. Polyethylenimine (PEI) was prepared as previously described (Smith K. et al., 2009) by dissolving 1 mg/ml PEI in 80ºC MilliQ water. It was left to cool down, and then the pH was adjusted to 7.2 with HCl. The solution was filter sterilized with a 0.45-mm syringe filter and stored at -20 ºC. The transfections

were performed as recommended (Durocher et al., 2002). Briefly, the procedure involves plating $2x10^5$cells per ml of medium (containing FBS) the day before transfection. The next day, the medium is poured off and replaced with 90% (of the total volume) fresh medium (containing FBS) and the cells are left in the incubator. 10% of the final volume is used for transfection mixture. To the transfection mixture, fresh medium (FBS free), is added DNA and vortexed for 1sec. The PEI is then added and vortexed for 1sec. The solution is left for 15 min at room temperature. After the incubation time, the transfection mixture is added to the cells and mixed to ensure an equal distribution of DNA/PEI complexes. The cells are left in the incubator overnight and the volume of medium doubled the day after. Transfected cells were usually left in the incubator for 10-14 days. IgE detection was confirmed by anti-IgE ELISA.

2.27 Anti-IgE ELISA

Anti-IgE ELISA was used for detection of IgE and measurement of its production levels during the optimization of transfection conditions. The ELISA was performed as previously described (McCloskey et al., 2007). Briefly, plates were sealed for all incubations at 37^{o}C, with the exception of overnight incubation, which was performed at 4^{o}C. Plate washings were performed using an Anthos Fluido ELISA plate washer (Jencons PLS), programmed for 4 washes with ELISA wash buffer (see table 2.2). For IgE detection, Nunc flat-bottomed ELISA plates were coated with 100 µl/well rabbit polyclonal anti-human IgE (1:7000 dilution) in ELISA carbonate buffer (see table 2.1.2) and incubated overnight at 4^{o}C. The plates were then flicked to remove the unbound coating antibody, blocked with 300 µl/well ELISA blocking buffer (see table 2.1.2) and incubated for 1 hour at 37ºC. Blocking buffer was removed by standard washing. Test samples were added to relevant wells in triplicate using appropriate dilutions (typically 1 in 10 and 1 in 20) in 1% Marvel-PBS-T20. The IgE standard (WHO) was added in 2-

fold dilutions (diluted in 1% Marvel-PBS-T20) down from 800 to 8 ng/ml, leaving the last well as blank. The plate was incubated for 2 hours at 37ºC, followed by standard washing. Bound material was detected by the addition of HRP-conjugated secondary anti-human IgE antibody (Sigma) diluted 1:2000 in 1% Marvel-PBS-T20 (100 µl/well, 2 hour at 37^{o}C). After washing, 50 µl/well of OPD Buffer (see table 2.1.2) were added and left for colour development in the dark at room temperature for around 15 minutes (or untill the yellow colour was vesible). Colour development was terminated by addition of 3 M HCl (50 µl /well). Absorbance of each well was measured at 492 nm using an automatic ELISA plate reader (Titertek Multiskan, Flow) linked to the Ascent software.

2.28 Processing Supernatants

Once IgE has been detected by Anti-IgE ELISA and the expression level was high enough to continue with the purification process after 10-14 days of incubation, the culture supernatants were collected and filtered using vacuum Stericup filter devices (Millipore). After addition of sodium azide (0.1% final concentration) the supernatants were left at 4ºC or directly applied to affinity chromatography.

2.29 Affinity chromatography

Purification of IgE isotype antibodies were performed using an IgG4-Fc-(sFcεRIα)$_2$ fusion protein (Shi et al., 1997). The fusion protein was expressed and purified as previously described (Shi et al, 1997) and amine coupled to 5ml HiTrap NHS-activated HP column (GE Healthcare) according to the manufacturer's instructions. All purifications were performed using an ÄKTA Prime system (Amersham, Uppsala Sweden)All affinity chromatography purifications of IgE were performed using an

automated apparatus fitted with a peristaltic pump (ÄKTA Prime), passing fluid over the column at 1 ml/min. The first steps involved washing the column with TBS, then passing the cell culture supernatant over the column to bind to FcεRIαγ. Captured antibody was then eluted with 0.2 M glycine, pH 2.5. The eluate was immediately neutralized with 1 M Tris, pH 8.6. The column was then re-equilibrated by cycling of PBS solution. In order to exclude any aggregated and/or degradation antibody products, size-exclusion chromatography was performed.

2.30 Size-exclusion chromatography

Size-exclusion chromatography was used for analysis and fractionation of the affinity column-purified antibodies. Fractionation was performed to isolate pure monomeric antibodies from oligomers and aggregated materials according to size. Aggregated molecules and contaminants passed through the porous bead matrix faster, followed by dimers, monomers, and finally, degradation products, which are trapped in the smaller pores of the matrix. Gel filtration was performed on a Gilson HPLC system using the Superdex™ 200 column (Amersham Pharmacia Biotech), which is suitable for separating proteins between 10 – 600kDa. Fractions were collected between 10 to 20 minutes.

2.31 Protein concentrating and buffer exchange

Fractions of monomeric, dimeric, and polymeric antibodies were pooled together, and concentrated using Amicon® Ultra-15 centrifugal filter devices (Millipore). The devices contain cellulose membranes with a molecular cut off at 30 kDa. Purified IgE preparations were spun in refrigerated centrifuge for 4000 rpm, until the antibody volume was concentrated to approximately 200 µl. Buffer exchange to PBS was performed by adding PBS onto the filter unit, and re-concentrating. Antibody

concentration (mg/ml) was measured with NanoDrop ND-1000 Spectrophotometer at 280 nm wavelength. Antibody purity was then assessed on SDS-PAGE.

2.32 Protein sample preparation for SDS PAGE

Prior to PAGE analysis, 3.1µl β-mercaptoethanol was added in 31µl 5xSDS-Sample Buffer (see Table 2.2) for SDS-PAGE in reducing conditions. The protein dye was added, and the samples were denatured at 90°C for 2 minutes. The samples were then loaded on a gel.

2.33 SDS-PAGE

Antibody purity was assessed on SDS-PAGE using the discontinuous buffer system (Laemmli 1970). An 8-15% gradient gel was prepared according to a standard recipe (Maniatis et al., 1982). Atto system gel apparatus was used. The apparatus was filled with 400ml 1xSDS-PAGE Running Buffer (see Table 2.2). 1 – 2 µg of the protein samples in 10 – 20 µl solution was loaded, alongside 10 µl of either SeeBlue ™ Plus2 pre-stained standard marker (Invitrogen), Spectra™ multicolor high range protein ladder (Fermentas) or the Protein ladder 10-250kDa (NEB). Samples were run at 120V, until the bromophenol dye was at the bottom of the gel plates. The plates were then separated, and the gel stained with Coomassie Blue staining solution (see Table 2.2) overnight before de-staining with Coomassie Blue destaining solution (see Table 2.2) for 20min for visualization of proteins.

2.34 Flow cytometry

For standard flow cytometric analysis 5 x 10^5 cells were used for each experiment. The cells were placed in 5ml FACS tubes and washed twice with 2mls FACS buffer. For

staining experiments, RBL-SX38 cells were resuspended in 200 µl of FACS buffer containing 1µg of IgE and left on ice for one hour. The tubes were then topped up with FACS buffer, followed by two washes with 2mls buffer. At last the buffer was removed and the tubes wiped out with paper towel. Cells were then incubated with anti-human IgE FITC (Dako), diluted 1:500 in FACS buffer (before each experiment), and left on ice for 45 minutes in dark. The secondary incubation was followed by two washes with 2mls buffer and the cells were then resuspended in 500 µl buffer. Collection of Flow Cytometry data was conducted using a FACS CaliburTM (BD Biosciences), with gating on live cells determined by forward versus side scatter, and events analysed using CELLQuest™ (BD Biosciences).

2.35 RNA Extraction from B cells

RNAlater buffer was removed from the cultured B cells and replaced with 600µl RLT lysis buffer (Qiagen) containing 1% β-mercaptoethanol (Sigma-Aldrich). The B cells were homogenised in the lysis buffer using a hand held cordless motor homogeniser (Anachem, Luton, UK). RNA was purified from the homogenate using Qiagen's RNeasy Protect Mini Kit; the homogenate was spun down for 3 minutes at 13000rpm in a microcentrifuge and the supernatant transferred to a fresh, RNase free tube. 600µl 70% ethanol (BDH, Poole, UK) was mixed with the supernatant and the sample, including any precipitate formed on ethanol addition, applied to an RNeasy mini column in a 2ml collection tube. The column was spun for 15 seconds at 10000rpm and the flow-through discarded. 700µl Buffer RW1 was then added to the column and spun through for 15 seconds at 10000rpm. The flow-through and the collection tube were discarded at this point and the column placed in a new 2ml collection tube. 500µl Buffer RPE was added to the column and spun through for 15 seconds at 10000 rpm. The flow-through was discarded and an additional 500µl Buffer RPE placed on the

column. This second wash was centrifuged for 2 minutes at 10000rpm to dry the column membrane. RNA was eluted from the column by addition of 40µl RNase free water and centrifuging for 1 minute at 10000rpm. RNA samples were stored at -70°C.

2.36 cDNA Synthesis

cDNA was generated from 0.5-5µg RNA using 400U Maloney Murine Leukaemia Virus Reverse Transcriptase (Invitrogen), 0.05ng random primers, 0.2ng oligo dT primers (both Promega), 0.4mM dNTPs (Invitrogen) and 8U RNase OUT (Invitrogen) in 1 x 1st strand buffer (Invitrogen) and 5mM DTT (Invitrogen). The reaction mix was heated to 70°C for 2 minutes before addition of the RT on ice. The whole reaction was then incubated at 37°C for 10 minutes, 42°C for 45 minutes and 50°C for 10 minutes. 160µl nuclease free water was added to the completed reaction and incubated for 2 minutes at 100°C to inactivate the RT before storage at -20°C.

3 TRANSIENT ANTIBODY EXPRESSION SYSTEM

3.1 Introduction

Over the last decade, antibody-based immunotherapies have been developed to treat a variety of diseases. This has required the selection of recombinant antibodies with high affinity for the appropriate epitopes on the target antigen and other desirable characteristics, such as the antibody isotype and effector functions. The antigen-binding variable (V) regions of the heavy- and light-chains, are in fact the products of three gene segments, namely the VH, DH, and JH, for the heavy-chain, and two gene segments, namely the VL and JL, for the light-chain. During expression of the heavy-chain, one of each of multiple germline VH, DH and JH gene segments are joined tail to head to form the final complete VH coding DNA (Janeway et al, 2001). Thus the heavy-chain V-region coding sequence can occur in various combinations of VDJ, which generates the combinational diversity of antibodies, together with the VL, which is assembled in the same way, except for the absence of the D- genes (Janeway et al, 2001).

Large cDNA libraries of the V gene segments of antibodies are frequently generated in the study of immune disorders that involve B-cells, such as autoimmune diseases (Foreman et al, 2007), allergy and asthma (Coker et al, 2003; Coker et al, 2005; Janezic et al, 1998; Snow et al, 1995; Snow et al, 1997; Snow et al, 1999) or cancer (Bende et al, 2002; Küppers et al, 1996; Tobin, 2005; Tobin et al, 2006). While this provides an insight at the genetic level, the antigen binding and effector function may only be elucidated from the expressed antibody. Similarly, large libraries of V gene segments may be generated during phage display. This is an *in-vitro* technology, in which the V genes are usually expressed as Fab or scFv antibody fragments and screened to identify antigen specificity (Hoogenboom, 2005). In this case, a method for rapid reformatting

as full-length antibodies would be highly beneficial in order to evaluate their biological function.

Conventional methods for recombinant expression of whole antibodies of interest typically requires the establishment of cell lines derived from CHO, mouse myeloma or PER.C6 cells (Bebbington et al, 1992; Filpula, 2007; Jain et al, 2007; Jones et al, 2003; Maynard & Georgiou, 2000; Zafir-Lavie et al, 2007). This tends to be a lengthy, low efficiency process involving extensive selection and screening and is consequently unfavourable for expressing large numbers of antibodies for functional studies, as may be required after generating a variable gene library. Therefore, our group aimed to design and develop a novel cloning methodology involving a cassette that allows exchange of both the variable and constant domain genes and facilitates the recombinant expression of a large number of antibodies. Tailoring the cassette for use in a vector suitable for transient transfection in mammalian HEK293E cells would enable rapid antibody production (Baldi et al, 2005; Berntzen et al, 2005; Li et al, 2007; Wright et al, 2003) in quantities sufficient to assay their biological characteristics. A HEK293E system for the expression of variable genes isolated from single cells e.g., human IgG1, has been described previously, which introduced restriction sites into the 5' and 3' ends of the variable domains of the heavy and light chain to enable ligation into vectors containing human Cγ1, Cκ1 or Cλ2 genes, as appropriate (Tiller et al, 2008). However, the restriction sites used are not compatible with all human constant gene isotypes. Moreover, a murine Ig gene secretary leader directed the protein secretion in each of these vectors. In attempt to tackle this problem, our group sought to generate a system with improved flexibility in which a human/chimeric antibody of any isotype and specificity could be expressed, utilising human IgH, Igκ or Igλ specific secretary leaders. Three cloning cassettes coding for either IgH, Igκ or Igλ chain expression have

been designed. In each cassette, a restriction site has been introduced by silent mutation into the junction between chain specific secretary leader and V gene. An additional restriction site was also introduced at the junction between V gene and C gene (Figure 3.1), to allow easy exchange of variable/constant domain genes by restriction enzymes and ligase-dependent cloning. For the heavy chain (IgH) cassette, BssHII restriction site was introduced by silent mutation into the V_H 1-02 secretary leader sequence, SacII was introduced into the Vκ A26 secretary leader sequence of the Igκ cassette and SalI was introduced into the Vλ8a leader sequence of the Igλ cassette. Similarly, NheI was introduced into V_H-C_H domain boundary, BsiWI into the Vκ-Cκ boundary and AvrII into the Vλ-Cλ boundary. The restriction site combinations are compatible with all human C gene isotypes and all known human and murine antibody framework regions.

The designed cassettes were subsequently ligated into vector pTT3 (Durocher et al, 2002) and the generated vectors named pSGH, pSGK and pSGL, coding for the expression of IgH, Igκ and Igλ, respectively (Figure 3.2). The final transient expression system thus employs a two-vector system (Fouser et al, 1992; Wood et al, 1990) or a co-transfection of pSGH and pSGK/pSGL vectors. The latter utilises polyethylenimine (PEI) (Boussif et al, 1995) as transfection reagent for DNA incorporation into HEK293E cells (Durocher et al, 2002).

A)

```
                M   D   W   T   W   R   I   L   F   L   V   A   A   A   T   G   A   H   S
VH 1-02:  ATG GAC TGG ACC TGG AGG ATC CTC TTC TTG GTG GCA GCA GCC ACA GGA GCC CAC TCC

1-02:     ATG GAC TGG ACC TGG AGG ATC CTC TTC TTG GTG GCA GCA GCC ACA GGC GCG CAC TCC
                                                                    BssHII
```

B)

```
                 M   L   P   S   Q   L   I   G   F   L   L   L   W   V   P   A   S   R   G
Vk lead A26:  ATG TTG CCA TCA CAA CTC ATT GGG TTT CTG CTG CTC TGG GTT CCA GCC TCC AGG GGT

A26:          ATG TTG CCA TCA CAA CTC ATT GGG TTT CTG CTG CTC TGG GTT CCA GCC TCC CGC GGT
                                                                        SacII
```

C)

```
                 M   A   W   M   M   L   L   L   G   L   L   A   Y   G   S   G   V   D   S
Vλ lead 8a:   ATG GCC TGG ATG ATG CTT CTC CTC GGA CTC CTT GCT TAT GGA TCA GGA GTG GAT TCT

8a:           ATG GCC TGG ATG ATG CTT CTC CTC GGA CTC CTT GCT TAT GGA TCA GGA GTC GAC TCT
                                                                        SalI
```

D)

```
                  V   T   V   S   S   A   S
JH-IgA2:      GTC ACC GTC TCY TCA GCA TCC

JH-IgE:       GTC ACC GTC TCY TCA GCC TCC

JH-IgG1:      GTC ACC GTC TCY TCA GCC TCC

JH-IgG2:      GTC ACC GTC TCY TCA GCC TCC

JH-IgG3:      GTC ACC GTC TCY TCA GCT TCC

JH-IgG4:      GTC ACC GTC TCY TCA GCT TCC

JH-IgH:       GTC ACC GTC TCG AGC GCT AGC
                                    NheI
```

E)

```
                 I   K   R   T   V   A   A   P   S
Jk-Ck:       ATY AAA CGA ACT GTG GCT GCA CCA TCT

Jk-Ck:       ATY AAA CGT ACG GTG GCT GCA CCA TCT
                     BsiWI
```

F)

```
                  T   V   L   G   Q   P   K   A   A   P   S   V   T
JL-CL:        ACC GTC CTM GGT CAG CCC AAG GCT GCC CCC TCG GTC ACT

JL-CL:        ACC GTC CTA GGT CAG CCC AAG GCT GCC CCC TCG GTC ACT
                     AvrII
```

Figure 3.1: Schematic representation of restriction sites within pSG vectors. Restriction sites positioned by silent mutations into the junctions between chain specific secretary leader and variable heavy (A), variable kappa (B) or variable lambda gene (C), and between variable genes and constant heavy (D), constant kappa (E) or constant lambda gene (F) to enable exchange of either variable or constant domain genes.

To demonstrate the capacity of our transient expression system to make fully functional antibodies, I have expressed and characterised previously cloned IgEs in our laboratory. To further establish the system's versatility to swap variable regions, I have cloned, expressed and characterised a High Molecular Weight Melanoma-Associated Antigen (HMW-MAA) specific IgE.

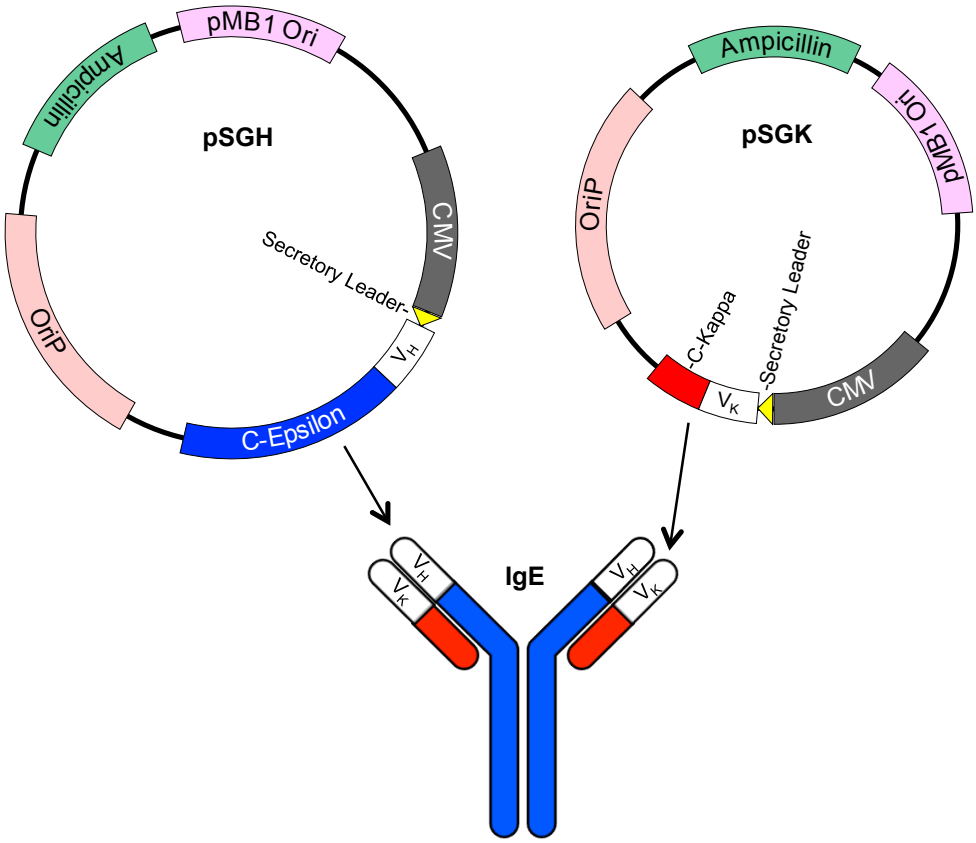


Figure 3.2: Schematic representation of pSGH and pSGK vectors. Transient expression vectors pSGH and pSGK consisting of pTT3 vector backbone, V_H 1-02 secretory leader and immunoglobulin Epsilon (blue) heavy chain expression cassette and Vκ A26 secretory leader and immunoglobulin Kappa (red) light chain expression cassette respectively, for the co-transfection and production of IgE.

It is accepted that antigen-cross-linked IgE triggers signalling, and activates mast cells and basophils through FcεRI aggregation. However, this long-held dogma was challenged in 2001, when two research groups working on rodent mast cells reported that monomeric IgE, in the absence of known antigen, was able to elicit mast cell

survival (Asai et al, 2001; Kalesnikoff et al, 2001). These IgE molecules exhibited different antigen-independent activities in promoting mast cell survival and cytokine production and were later named cytokinergic IgEs. Based on the cytokinergic activity elicited by the IgE molecules, an operational classification of 'highly cytokinergic' (HC) and 'poorly cytokinergic' (PC) was used (Kitaura et al, 2003). The cytokinergic IgE phenomenon has not been a part of my studies and is therefore not reported in this thesis. However, as cytokinergic IgEs have been cloned, expressed and characterised in our laboratory (Gan, 2008), I have used them as a model for my studies and as a standard for further optimisations of the transient antibody expression system.

3.2 Methods

3.2.1 Transient transfection and expression of cytokinergic IgEs

pSGH and pSGK/pSGL vectors coding for the expression of cytokinergic IgEs (Gan, 2008) were transformed into XL1-Blue competent cells (Stratagene) according to the manufacturer's instructions. Single colonies were picked for each clone and inoculated in 5mls LB broth (Invitrogen) supplemented with 100μg/ml ampicillin (Sigma). After overnight culture at 37°C with shaking at 200rpm, bacteria were harvested and plasmid DNA purified with a Wizard Plus SV Minipreps DNA Purification System (Promega). Following sequencing confirmation of the heavy and light chain expression cassettes using pTT3-F and pTT3-R primers (Table 2.2), each confirmed colony was inoculated in 500ml LB broth containing 100μg/ml ampicillin and cultured overnight at 37°C with shaking at 200rpm. The plasmid DNA was purified from the inoculated cultures using a HiSpeed Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions.

The human embryonic kidney cell line HEK293E (ATCC no CRL-1537) was seeded at 2 x 10^5 cells per ml in DMEM High Glucose supplemented with 10% FCS, 2mM

glutamine 10U penicillin / streptomycin and 250μg/ml Geneticin (all Invitrogen) onto 500cm^2 triple layered flasks (Nunc) to a final volume of 250ml and cultured overnight at 37°C, 5% CO_2. Cells were transiently transfected following S. Gan's protocol using a 2:1 ratio of polyethylenimine (PEI) (Sigma) to DNA and a 4:1 ratio of pSGK/pSGL (expressing kappa/lambda light chain) to pSGH (expressing heavy chain). To produce 0.5-4mg of each cytokinergic IgE antibody (based on previously reported expression levels), four 500cm^2 triple layered flasks with adherent seeded cells had to be transfected for a final volume of 1L. For each triple layered flask, 500μg PEI was mixed with 200μg pSGK/pSGL and 50μg pSGH in 15ml serum free DMEM and incubated for 15 minutes at room temperature. Media from the overnight culture was replaced with 235ml fresh DMEM High Glucose plus 10% FCS, 2mM glutamine 10U penicillin / streptomycin and 250μg/ml Geneticin and topped up to 250ml with the pre-incubated 15ml transfection mix. After a second overnight culture, the media was again replaced with 250ml fresh DMEM High Glucose supplemented with 10% FCS, 2mM glutamine 10U penicillin / streptomycin and 250μg/ml Geneticin and the cells were left in culture for antibody expression. Following 20 days incubation, the media was harvested and centrifuged at 1000 x g for 15 minutes to pellet cell debris. Supernatants were passed over 0.45μm filters (Sartorius) and stored at 4°C with 0.1% sodium azide (Sigma) till further analysis/use.

3.2.2 Affinity purification

IgEs were purified on AKTA prime system. The affinity column was equilibrated with 10 CV (Column Volumes) of phosphate buffered saline (PBS) washing buffer (pH 7.0, 0.45 μm filtered). The supernatants were loaded onto the column at a flow rate of 2 ml/min and the column washed with 10 CV washing buffer. Captured IgE was eluted with 0.2M Glycine, pH 2.5 and 2.5 ml fractions were collected into tubes containing 0.5

ml 1M Tris–HCl pH 8.6 for neutralisation. The column was re-equilibrated with 10 CV washing buffer.

3.2.3 Gel filtration

Gel filtration was performed on a Gilson HPLC system using a Superdex™ 200 10/300 GL column (Amersham, Uppsala Sweden), suitable for purifying proteins between 10 – 300kDa, at a flow rate of 0.75 ml/min in PBS pH 7.0 and fractions collected between 10 to 30 minutes.

3.2.4 Cloning of HMW-MAA IgE expression constructs

Previously described high-affinity murine IgG2a monoclonal antibody 225.28S (Natali et al, 1984) specific for the HMW-MAA, has been cloned in single-chain Fv configuration (scFv) for soluble expression in bacteria, showing conserved binding specificity of the parental antibody (Neri et al, 1996). The specific DNA antibody variable sequences for the heavy and light chains published by Neri *et al* (1996) were analysed using sequence alignment software (http://www.imgt.org/IMGT_vquest/share/textes/) for alignment of the V- J genes and allele identification. Once the first and last amino acids of the variable regions have been identified, restriction sites (according to our pSGH and pSGK cassettes) were designed to flank the variable sequences for subsequent cloning. BssHII restriction site (present in the V_H 1-02 leader in pSGH vector) has been integrated together with the rest of VH 1-02 leader at the N-terminal of the variable heavy chain region and NheI restriction site integrated at the C-terminal. The variable light chain region was treated equally integrating SacII site (present in the Vκ A26 leader in pSGK vector) together with the rest of VK A26 leader at the N-terminal of the variable light chain region and BsiWI at the C-terminal. The amino acid sequence corresponding to variable heavy chain region (5'-AHSQVKLQQSGGGLVQPGGSMKLSCVVSGFTFSNYWMNWVRQSPEKGLEWI

AEIRLKSNNFGRYYAESVKGRFTISRDDSKSSAYLQMINLRAEDTGIYYCTSYG NYVGHYFDHWGQGTTVTVSSAS-3') and variable light chain region (5'-RGDIELTQSPKFMSTSVGDRVSVTCKASQNVDTNVAWYQQKPGQSPEPLLFSA SYRYTGVPDRFTGSGSGTDFTLTISNVQSEDLAEYFCQQYNSYPLTFGGGTKLEI KRT-3') were then synthesised (Geneart, Germany) using human codon optimisation. 5µg of plasmid DNA, containing the variable heavy and light chain regions ligated in ampicillin-resistant holding vectors from Geneart were diluted into 50 µl MilliQ water. 1 µl of the diluted DNA was transformed into XL1-Blue Competent Cells (Stratagene) according to the manufacturer's instructions. Plasmid DNA from overnight cultures was purified using a Wizard Plus SV Minipreps DNA Purification System (Promega) and subjected to restriction digests (New England Biolabs). 2 µg of variable heavy region DNA was initially digested with 10 units NheI for 2 hours in 25 µl total reaction volume and subsequently digested with 10 units BssHII for 2 hours in 50 µl total reaction volume according to the manufacturer's instructions. Simultaneously, 2 µg of variable light region DNA was initially digested with 10 units SacII for 2 hours in 25 µl total reaction volume and subsequently digested with 10 units BsiWI for 2 hours in 50 µl total reaction volume. The digested DNA was resolved on a 1% SeaKem Agarose gel and gel purified using Wizard® SV Gel and PCR Clean-Up System (Promega). Meanwhile, 2 µg of the expression vectors pSGH (expressing heavy chain) and pSGK (expressing light chain) were digested with NheI-BssHII and SacII- BsiWI respectively. The digested pSG vectors were heat inactivated for 20 minutes at 80ºC and treated with Antarctic Phosphatase (New England Biolabs) according to the manufacturer's instructions, following another heat inactivation for 5 minutes at 65ºC. The Antarctic Phosphatase removes 5' phosphates from the digested DNA, and thus prevents recircularisation of cloning vectors. The purified variable region DNAs and linearised pSGH and pSGK were then ligated utilising the Quick Ligation Kit (New England

Biolabs) according to manufacturer's instructions and 2 µl of each ligation reaction were transformed into XL2-Blue Ultracompetent Cells (Stratagene). Single colonies were picked for each clone and inoculated in 5mls LB broth (Invitrogen) supplemented with 100 µg/ml ampicillin. After overnight culture at 37°C with shaking at 200rpm, bacteria were harvested and plasmid DNA purified with a Wizard Plus SV Minipreps DNA Purification System (Promega). Following sequencing confirmation of the heavy and light chain expression cassettes using pTT3-F and pTT3-R primers, the generated vectors were named pSGH-MAA and pSGK-MAA. Each confirmed colony was subjected to Maxi preparations (see section 3.2) and the purified plasmid DNA used for subsequent transfections.

3.2.5 Flow Cytometry

Recombinant IgE was incubated with each cell line (A375 melanoma cells, primary human melanocytes, and RBL cells expressing both human and rat FcεRI (Dibbern et al, 2003)) for 30 min at 4^{o}C, followed by two washes in PBS supplemented with 5% normal goat serum (FACS buffer). Cells were then incubated with anti-human IgE-FITC (10 µg/mL) for 30 min at 4^{o}C and washed with FACS buffer prior to acquisition and analysis on a FACSCanto^{TM} (BD Biosciences)

3.3 Results

3.3.1 Transient expression of cytokinergic IgEs

The transient antibody expression system has been shown to produce functional antibodies (Gan, 2008). However, the antibody expression levels have been insufficient to support animal model experiments for comparative functional studies of different antibody isotypes. To increase the antibody expression levels, as one of the main goals of my project, I was given the opportunity to study the system by transiently expressing

the cytokinergic IgEs. Given that the average expression levels of these antibodies have already been measured (Gan, 2008), the main aim of this part of the project was to obtain the previously expressed levels and if possible, exceed them. Concentrations of expressed IgEs (Figure 3.3) in culture supernatants were determined by anti-human IgE ELISA as described previously (McCloskey et al, 2007). As the expression levels of all tested cytokinergic IgEs, confirmed by ELISA, matched the previously reported expression levels, it enabled me to continue with the purification process and optimise the system for better yields.

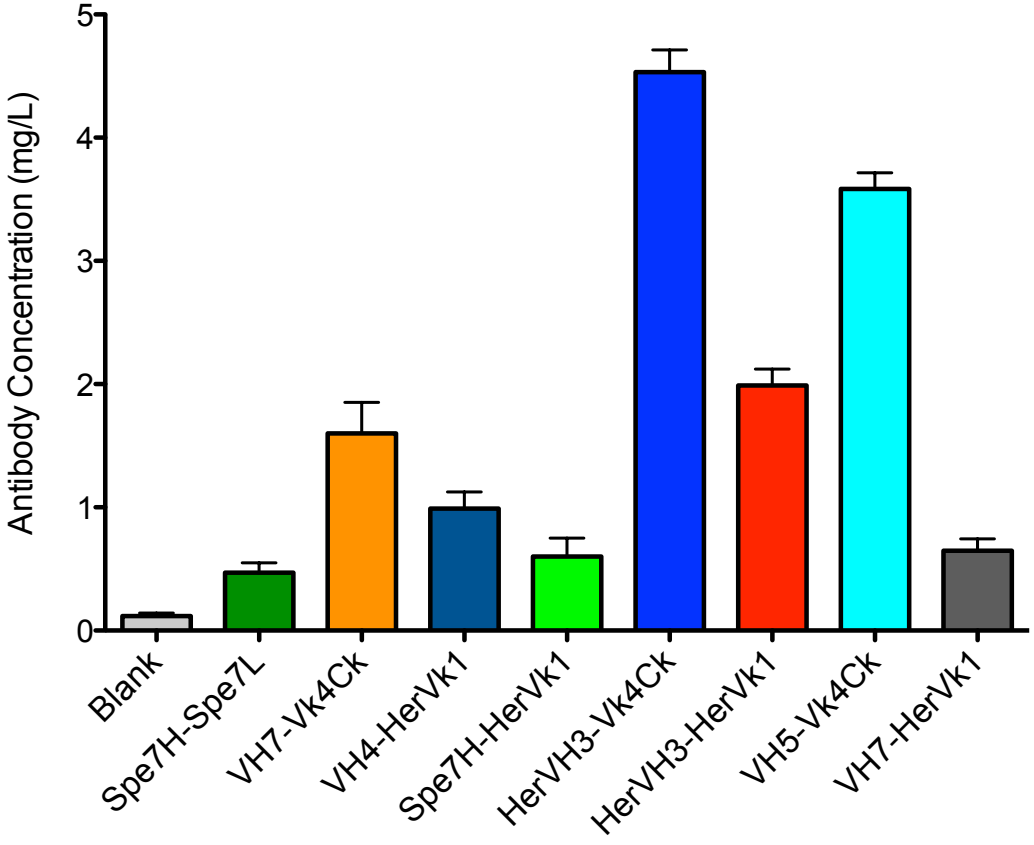


Figure 3.3: Quantification of transiently expressed IgEs. Anti-human IgE ELISA result-based comparison of antibody expression yields from transiently transfected HEK293E cells. The antibody concentration at mg/L was determined by reference to a standard curve and the results represent the mean of triplicate readings ± SD.

3.3.2 Optimisation of PEI : DNA ratio

Ideally, to reach the highest antibody productivity, all cells should uptake and express the transgene DNA with minimal toxicity (e.g., all cells should survive after transfection) caused by the transfecting agent. Polyethylenimine (PEI) has previously been utilised for large-scale transfection in studies using HEK293E cells (Pham et al, 2003). PEI was also characterised to have low cell toxicity and rapid efficient uptake by cells (Godbey et al, 2000; Godbey et al, 1999a; Godbey et al, 1999b; Godbey et al, 1999c; Godbey et al, 1999d). For the purpose of this project, PEI also provided the option of scaling up the production of antibody, without being prohibitively costly. Although a 2:1 ratio of PEI : DNA

of VH4 heavy chain and HerVk1 light chain, respectively) DNA concentration constant at 1 μg/ml, was further carried out. HEK293E cells were transiently transfected with 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1 and 8:1 ratios of PEI : DNA and sample supernatants collected 5 days post-transfection for anti-human IgE ELISA analysis as described previously (McCloskey et al, 2007). Simultaneously, the cells were trypsinised and the cell viability estimated using Trypan blue exclusion method (Figure 3.4). Optimum cell viability and maximum antibody expression level within 5 days incubation period was found to be the best at 4μg PEI : 1μg DNA ratio.

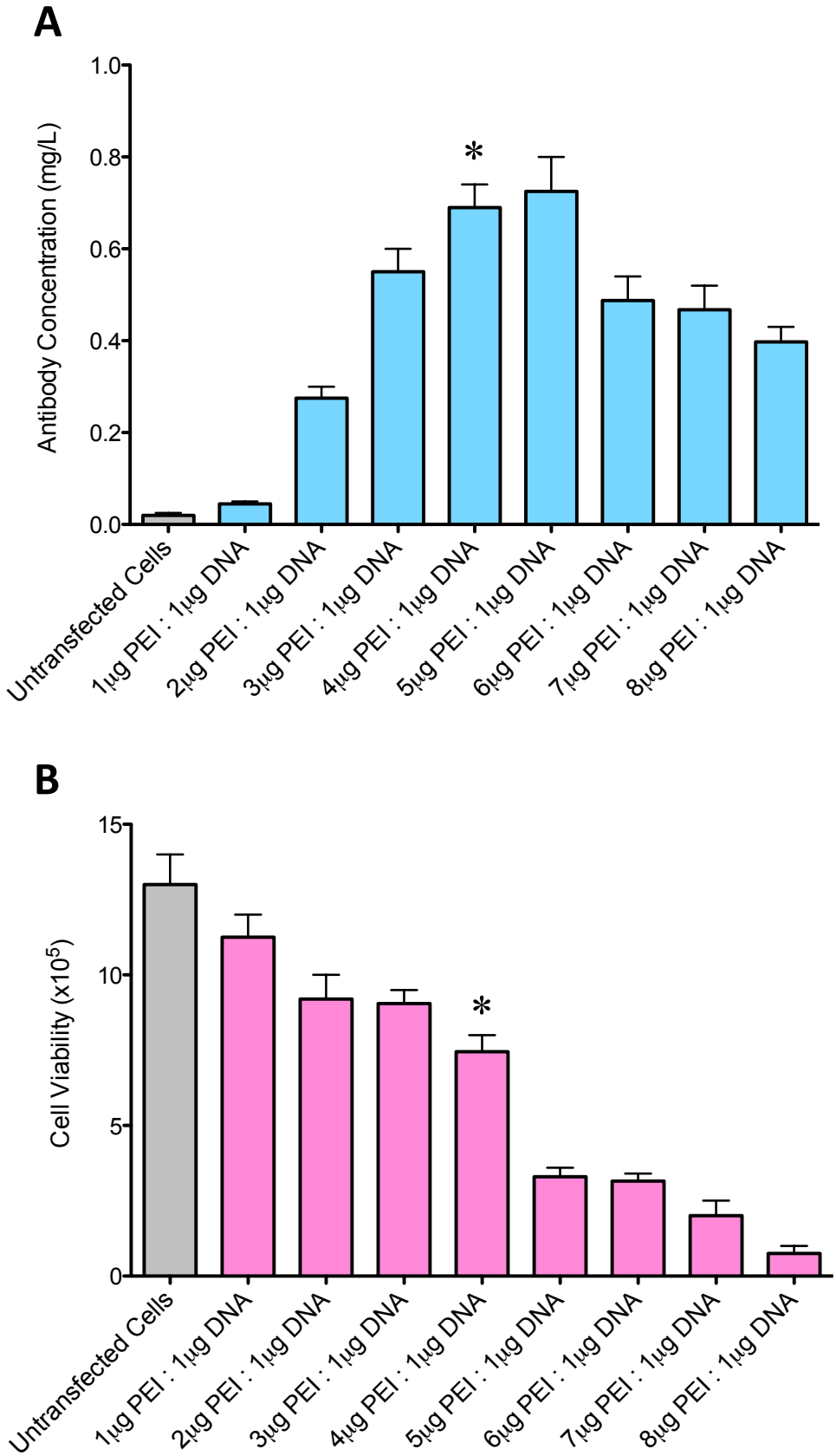


Figure 3.4: Optimisation of PEI:DNA ratio for transient expression of antibodies. (A) Anti-human IgE ELISA result-based comparison of antibody expression yields. The antibody concentration at mg/L was determined by reference to a standard curve and the results represent the mean of triplicate readings ± SD. (B) Cell viability determined by cell count using Trypan blue exclusion method 5 days post-transfection.

3.3.3 Optimisation of light : heavy chain DNA ratio

Previous studies (Baldi et al, 2005; Schlatter et al, 2005) have shown an important role of light-chain expression in Ig production, suggesting a higher light-chain expression increases antibody production yield. To investigate this phenomenon and further boost the antibody production, optimisation of transfection conditions with HEK293E was then carried out to determine the most effective light : heavy chain ratio. HEK293E cells were transiently transfected with increasing concentrations of pSG-HerVk1 (HerVk1 light chain) DNA, whilst keeping the pSG-VH4 (VH4 heavy chain) DNA concentration constant, limiting the total DNA concentration to 1 μg per ml of culture media, with previously optimised 4:1 ratio of PEI : DNA. Sample supernatants were collected 10 days post-transfection and the concentrations of IgE expression in culture supernatants (Figure 3.5) were determined by anti-human IgE ELISA as described previously (McCloskey et al, 2007).

The ELISA analysis showed the highest antibody expression yield was present in cultures transfected with a 2:1 ratio of light : heavy chain DNA. This optimisation halved the DNA amount of the light chain previously used (4:1) to transfect cells, hence shortening the DNA production process. Therefore, subsequent HEK293E transfections were performed using 2:1 ratio of light : heavy chain DNA.

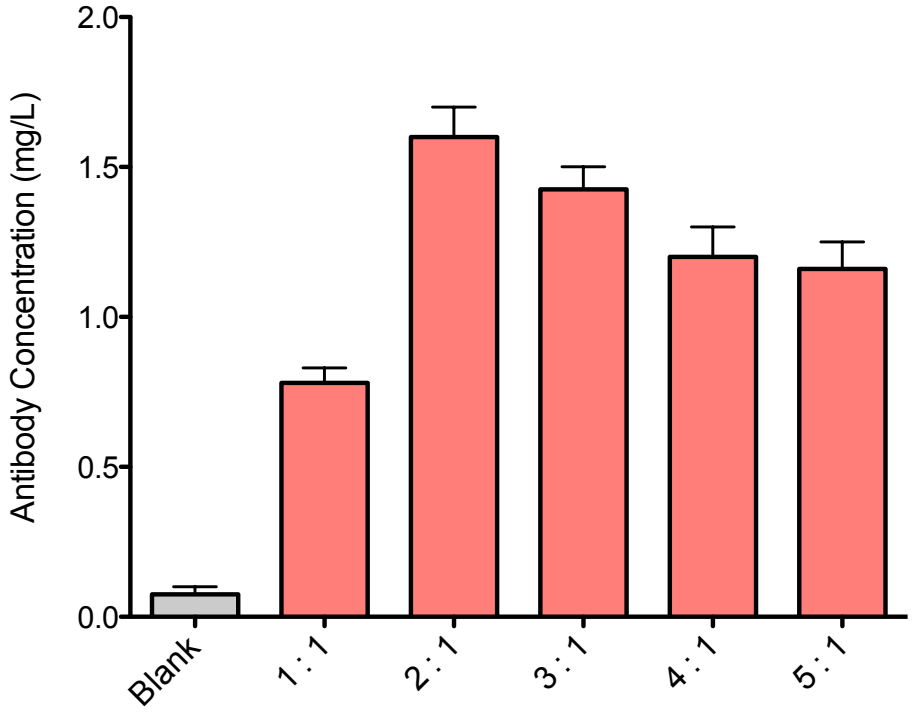


Figure 3.5: Optimisation of Light : Heavy chain DNA ratio for transient expression of antibodies. Anti-human IgE ELISA result-based comparison of antibody expression yields after 10 days incubation of transiently transfected HEK293E cells with increasing concentrations of light chain DNA. The antibody concentration at mg/L was determined by reference to a standard curve and the results represent the mean of triplicate readings ± SD.

3.3.4 Optimisation of media supplements

Despite the successful use of HEK293E cells for antibody expression by large-scale transfection, the labour intensive procedure and the high cost / low yield characteristics of mammalian cell culture processes remain an important drawback. Therefore, I sought to optimise various production parameters and to increase the volumetric antibody productivity. Previous studies (Pham et al, 2005) reported the addition of Tryptone N1 (a casein peptone, TN1) to the cell culture media 24 h post-transfection (hpt), led to a 2-fold increase in protein production after 5 days incubation. In addition, an Invitrogen product called Optimab, a monoclonal antibody production enhancer, has been previously shown to increase antibody production. I strove to maximise antibody yields following large-scale transfection by supplementing the culture media with Tryptone N1 and Optimab. HEK293E cells were transiently transfected with pSG-HerVk1

(HerVk1 light chain) and pSG-VH4 (VH4 heavy chain) with previously optimised 2:1 ratio of light : heavy chain DNA and 4:1 ratio of PEI : DNA, following 24h post-transfection supplementation of the culture media with Optimab or Tryptone N1. Sample supernatants were collected after 12 days incubation and the antibody expression levels (Figure 3.6) were determined by anti-human IgE ELISA as described previously (McCloskey et al, 2007).

The ELISA analysis showed a 4-fold decrease in antibody expression level in the cultures supplemented with Tryptone N1, suggesting the peptone depletion was not limiting antibody production yield in HEK293E cells (Figure 3.6). However, the Optimab supplementation represented a 2-fold increase in volumetric antibody productivity obtained 12 days post-transfection.

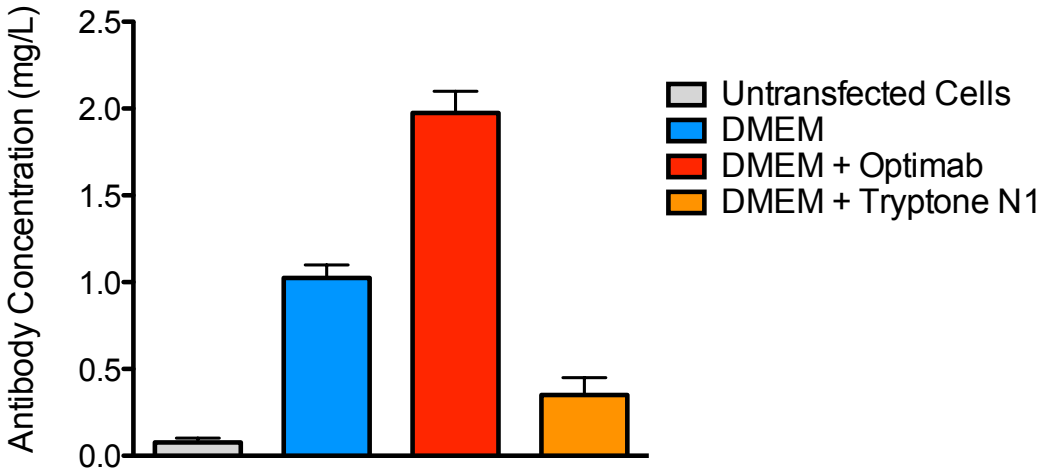


Figure 3.6: Optimisation of media supplements for transient expression of antibodies. Anti-human IgE ELISA result-based comparison of antibody expression yields from transiently transfected HEK293E cells, following 24h post-transfection media supplementation with Optimab or Tryptone N1. The antibody concentration at mg/L was determined by reference to a standard curve and the results represent the mean of triplicate readings ± SD.

3.3.5 Purification of IgE antibodies

The transiently expressed IgEs antibodies were purified by affinity chromatography using IgG4-Fc-(sFcεRIα)$_2$ fusion protein, a soluble fragment of the high-affinity IgE receptor R-chain fused to the Fc region of IgG4. The fusion protein was expressed and purified as previously described (Shi et al, 1997) and amine coupled to 5ml HiTrap NHS-activated HP column (GE Healthcare) according to the manufacturer's instructions. All purifications were performed using an ÄKTA Prime system (Amersham, Uppsala Sweden) (Figure 3.7).

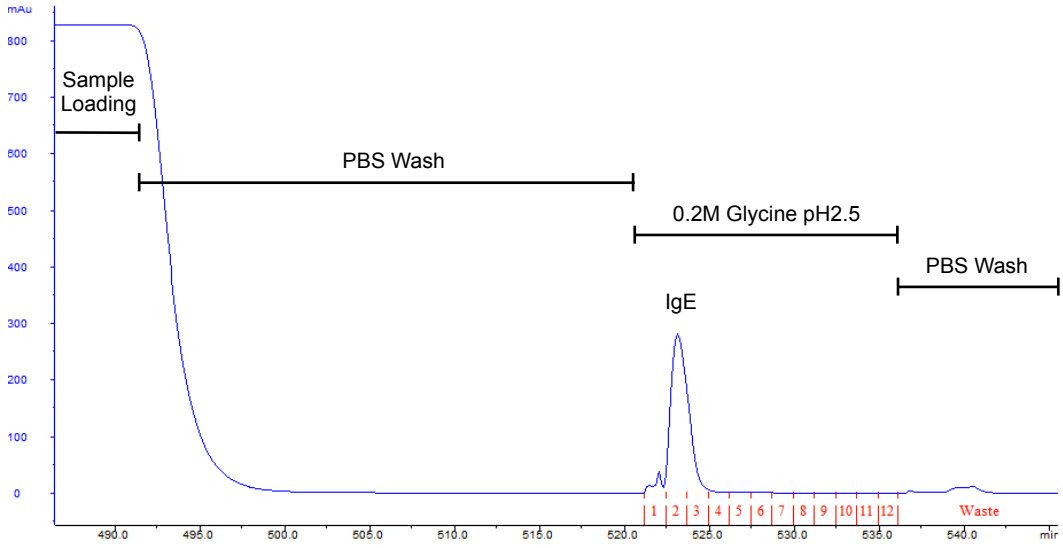


Figure 3.7: Affinity chromatography analysis of transiently expressed IgEs. Elution profile from affinity purification of transiently expressed IgE on a IgG4-Fc-(sFcεRIα)$_2$ fusion protein column by AKTA Prime. IgE is eluted by low pH in the 2nd and 3rd fractions.

3.3.6 Size-exclusion chromatography of affinity purified IgEs

Gel filtration/size-exclusion chromatography is a common polishing step, following affinity purification of recombinant antibodies. However, during purification of some IgE antibodies, aggregation was observed, where certain combinations of IgE displayed aggregative tendencies. Therefore, size-exclusion chromatography was used for analysis

and fractionation of the affinity column-purified IgEs as previously described (Hunt et al, 2005). Fractionation was performed to isolate pure monomeric antibodies from aggregated materials according to size. Aggregated molecules passed through the porous bead matrix faster, followed by monomers, which are trapped in the smaller pores of the matrix. The size-exclusion chromatography analysis confirmed the aggregation observed in some samples as a very small peak eluted between 12th and 14th minute. To purify monomeric IgE antibodies, only fractions eluted between 14th and 16th minute were collected (Figure 3.8).

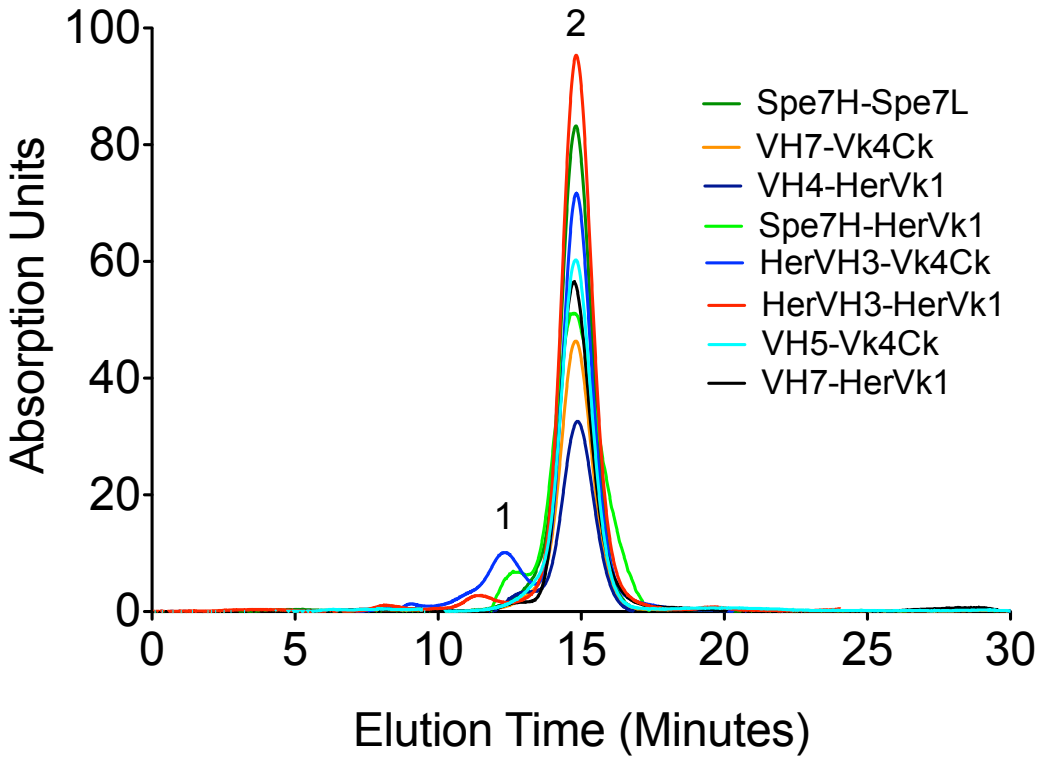


Figure 3.8: Size-exclusion chromatography analysis of affinity purified IgEs. Elution profile of affinity purified IgE antibodies from Superdex™ 200 gel filtration. Single peaks (1) corresponding to aggregated products in some antibodies eluted between 12th and 14th minute from the start of the run; monomeric antibodies eluted between 14th and 16th minute.

3.3.7 SDS-PAGE analysis of affinity purified IgEs

Monomeric antibodies isolated from the size-exclusion chromatography were concentrated using Amicon® Ultra-15 centrifugal filter devices (Millipore) and subjected to Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE). The SDS-PAGE of samples with identical charge per unit mass due to binding of SDS results in fractionation by size. 2µg of each purified IgE were loaded on 4-15% gradient gels under reducing and non-reducing conditions (Figure 3.9). Under non-reducing conditions (Figure 3.9A), the purified IgEs were found as single bands between 180 kDa and 250 kDa of the Spectra™ multicolor high range protein ladder corresponding to the expected ~190 kDa whole IgE molecule. However, under reducing conditions (Figure 3.9B), single bands were found at 25 kDa of the Protein ladder 10-250kDa (NEB) corresponding to the antibody light chains (25 kDa) and single bands found at 80 kDa possibly corresponding to glycosylated forms of the antibody heavy chains (~60 kDa).

None of the purified antibodies showed significant contamination with other proteins, as judged by the absence of bands of molecular weight inconsistent with those expected for an IgE antibody polypeptide chain. This demonstrated that the affinity column purification and size-exclusion chromatography were effective in yielding pure monomeric IgEs.

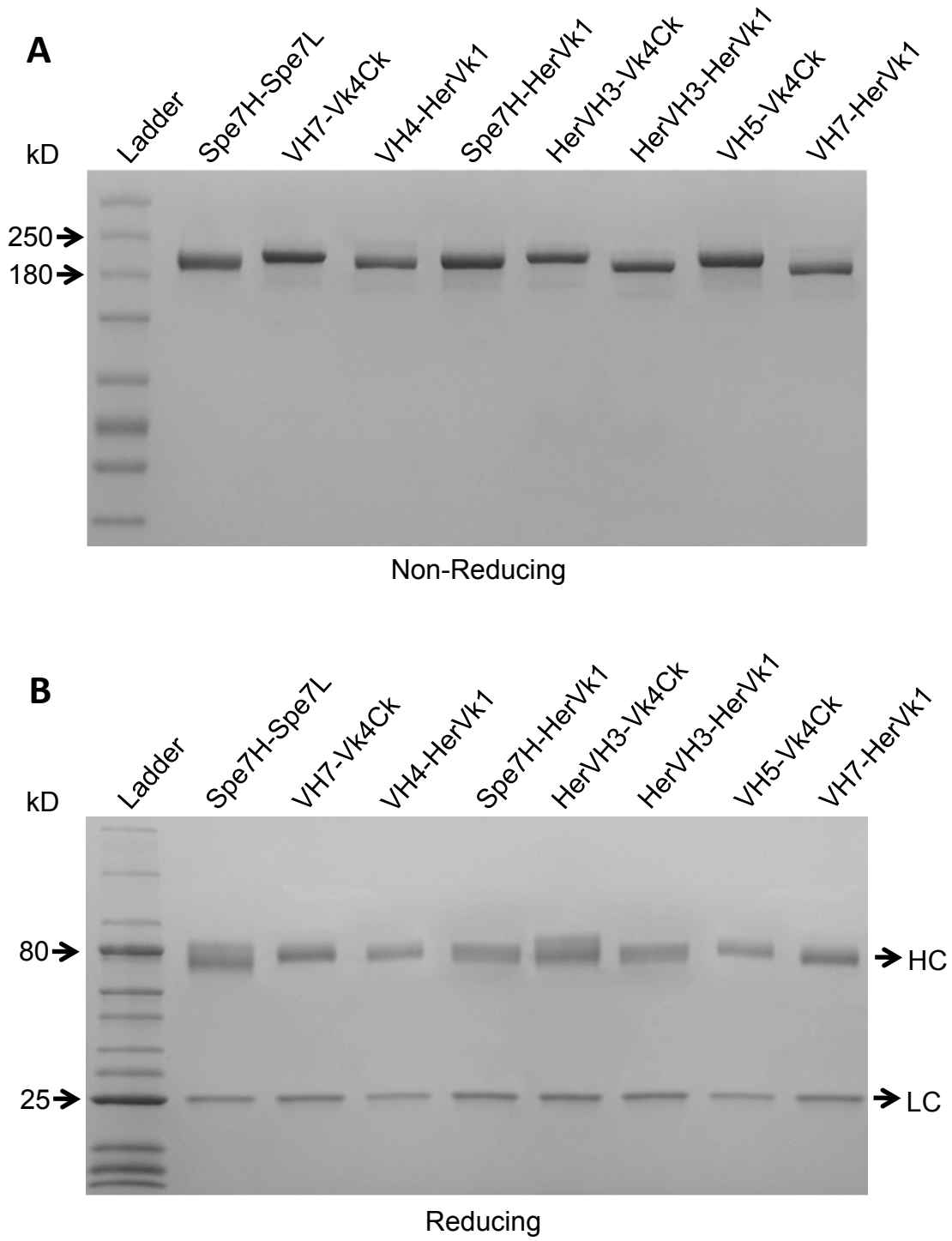


Figure 3.9: SDS-PAGE analysis of affinity purified IgEs. Affinity purified antibodies analysed under reducing and non-reducing conditions and visualised by Coomassie staining. (A) Antibodies analysed under non-reducing conditions, alongside with Spectra™ multicolor high range protein ladder, showing a single band corresponding to the ~190 kDa expected to whole IgE molecule. (B) Antibodies analysed under reducing conditions, alongside with Protein ladder 10-250kDa (NEB), showing 25kDa light chains and glycosylated heavy chains.

3.3.8 Receptor-binding activity of affinity purified IgEs

To assess the functionality of the transiently expressed and affinity-purified antibodies, I tested the receptor-binding activity of IgEs. For the purpose, RBL-SX38 cells expressing both human and rat FcεRI (Dibbern et al, 2003) were incubated with the purified IgEs and subsequently stained with anti-human IgE FITC (Dako). Collection of flow cytometry data was conducted using a FACS Calibur™ (BD Biosciences), with gating on live cells determined by forward versus side scatter, and events analysed using FlowJo (Treestar). The flow cytometry analysis (Figure 3.10) of RBL-SX38 cells incubated with three of the purified IgEs shows a clear right shift of fluorescence intensity, indicating the functional receptor-binding activity of the recombinant antibodies.

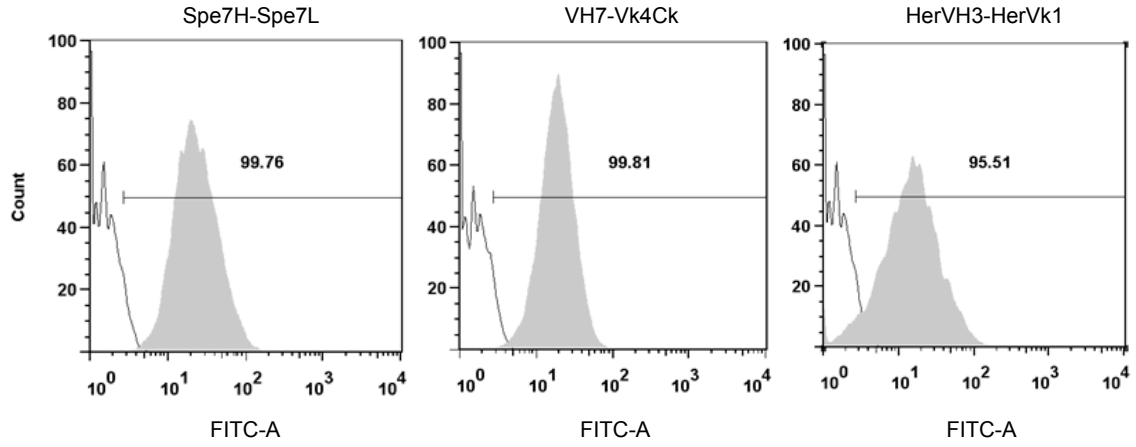


Figure 3.10: Receptor-binding activity of affinity purified IgEs. Flow cytometric histograms showing fluorescent intensity of RBL-SX38 cells incubated with affinity purified IgEs plus FITC-labelled anti-human IgE (filled in grey) and RBL-SX38 cells alone (not filled) against the number of cells. The IgE-Fc fragments of the purified antibodies demonstrate effector-binding to RBL SX38 mast cells, expressing human FcεR1 receptor.

3.3.9 Swapping variable regions within pSG vectors

The superiority of the IgE isotype over IgG1 in cancer immunotherapy has been demonstrated in murine models (Gould et al, 1999; Karagiannis et al, 2003). We aim to test this hypothesis further by developing IgE isotypes of melanoma-specific and other anti-tumour antibodies (described in details in Chapter 5). The first example, also used as a control for our antibody discovery program (described in details in Chapter 6), was the conversion of an existing IgG against a melanoma-specific antigen, the High Molecular Weight Melanoma-Associated Antigen (HMW-MAA), into IgE.

Having successfully expressed, purified and characterised the previously cloned cytokinergic IgEs, I strove to study the versatility of the cloning cassette to exchange variable regions and to demonstrate the capacity of the expression system to make fully functional antibodies. As an example, I have exchanged the existing V genes from pSGH and pSGK with those specific for the HMW-MAA, utilising the BssHII and NheI restriction sites in the heavy chain cassette and SacII and BsiWI in the light chain cassette (Figure 3.11). The sequencing confirmation of the newly generated pSGH-MAA and pSGK-MAA vectors demonstrated an effective exchange of the variable genes within pSG vectors using restriction enzymes and ligase-dependent cloning.

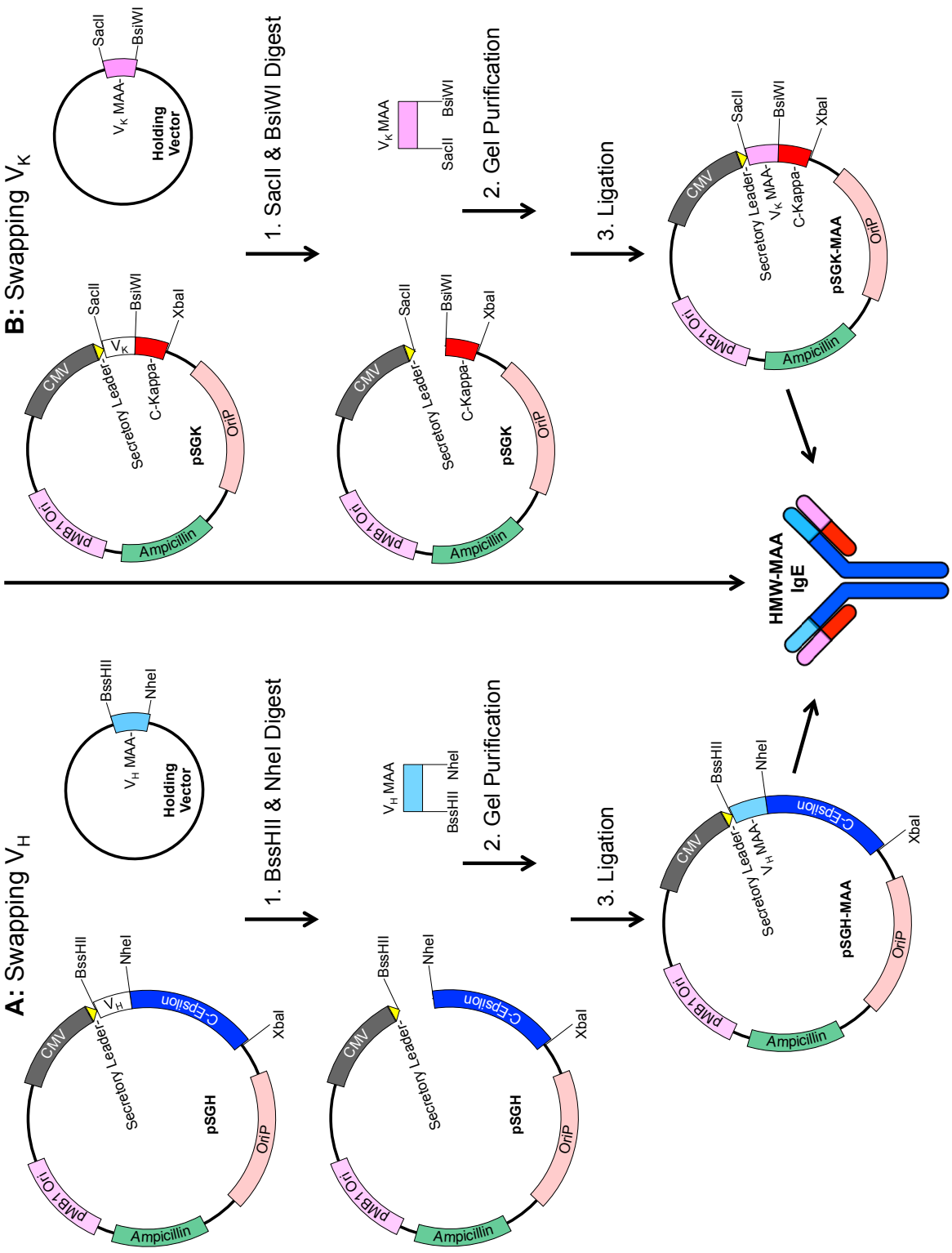


Figure 3.11: Schematic representation of conventional antibody cloning into pSG expression vectors. Restriction enzymes and ligase-dependent cloning procedure of HMW-MAA specific antibody variable regions into pSGH and pSGK vectors. Swapping of variable heavy (A) and variable kappa regions (B) by double digest of pSG and HMW-MAA holding vectors with unique restriction enzymes, followed by gel purification of variable genes and ligation of linearised pSG vectors with gel purified variable genes. Production of HMW-MAA specific IgE is achieved by co-transfection of pSGH-MAA and pSGK-MAA in HEK293E cells.

3.3.10 Transient expression of HMW-MAA specific IgE

Following successful exchange of variable genes within pSG vectors, I proceeded with transfection and transient expression of HMW-MAA specific IgE antibody. HEK293E cells were transiently transfected with pSGH-MAA and pSGK-MAA with the previously optimised 2:1 ratio of light : heavy chain DNA and 4:1 ratio of PEI : DNA as described previously. Sample supernatants were collected 5 days post-transfection and the antibody expression levels were determined by anti-human IgE ELISA as described previously (McCloskey et al, 2007). The ELISA analysis showed the HMW-MAA specific IgE antibody was expressed at 0.7mg/L after 5 days incubation period (Figure 3.12). The result demonstrated successful transfection of HEK293E cells with the newly generated pSGH-MAA and pSGK-MAA vectors, and the capacity of the expression system to produce antibodies.

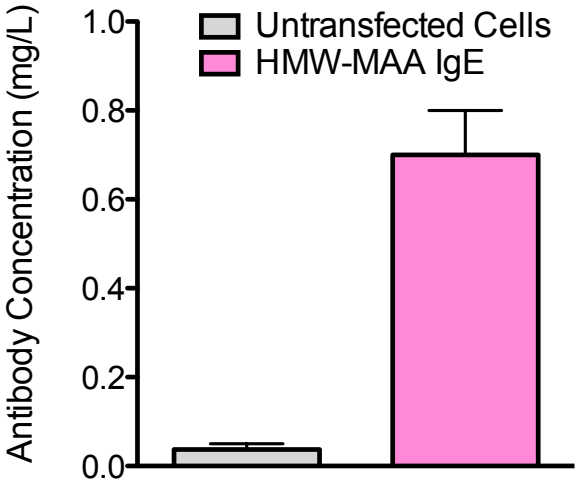


Figure 3.12: Quantification of transiently expressed HMW-MAA specific IgE. Anti-human IgE ELISA analysis of HMW-MAA specific IgE transient expression in triple layer flask. The antibody concentration at mg/L was determined by reference to a standard curve and the results represent the mean of triplicate readings ± SD.

3.3.11 Flow cytometric assessment of transiently expressed HMW-MAA specific IgE

To assess the functionality of the transiently expressed recombinant HMW-MAA specific IgE antibody, antigen specificity and receptor-binding activity were investigated by flow cytometry (Figure 3.13). Antigen specificity was analysed by selective binding to native HMW-MAA on the cell surface of A375 melanoma cells as opposed to primary human melanocytes, normally present in the skin of healthy patients. The receptor-binding activity of the antibody was analysed by binding to RBL cells which express both human and rat FcεRI (Dibbern et al, 2003). Flow cytometric analysis (Figure 3.13) of A375 melanoma cells incubated with the HMW-MAA specific IgE antibody, shows a clear right shift of fluorescence intensity, indicating preserved specific binding to native HMW-MAA and no binding above background to primary melanocytes. Furthermore, the IgE bound to human FcεR1 receptor, expressed on RBL SX38 mast cells, demonstrating functional receptor-binding activity of the recombinant antibody.

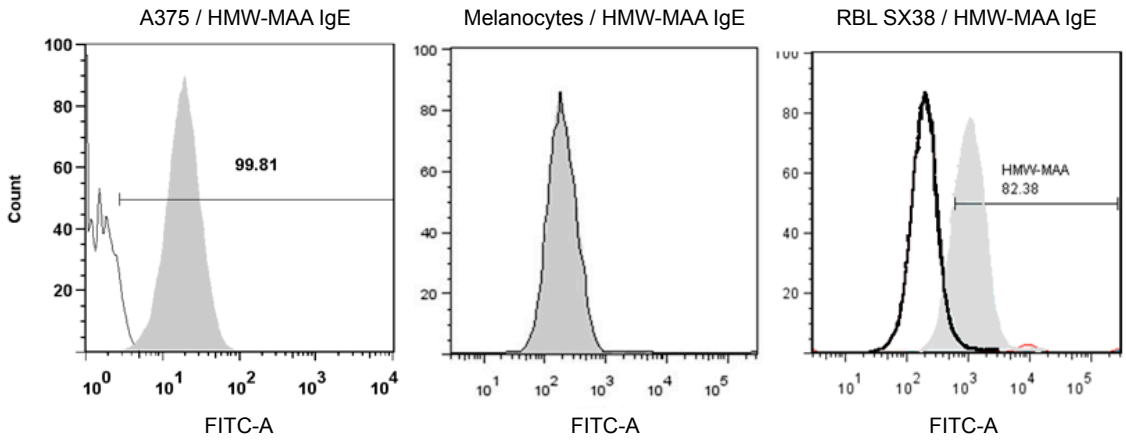


Figure 3.13: Flow cytometric assessment of transiently expressed HMW-MAA specific IgE. HMW-MAA specific IgE antibody shows specific binding to native HMW-MAA present on A375 melanoma cells and no binding above background to primary melanocytes. The IgE-Fc fragment of the antibody demonstrate effector-binding to RBL SX38 mast cells, expressing human FcεR1 receptor.

3.4 Discussion

To study the functional properties of antibody variable gene libraries, which are frequently generated in the study of B-cell associated immune diseases (Bende et al, 2002; Coker et al, 2003; Coker et al, 2005; Foreman et al, 2007; Janezic et al, 1998; Küppers et al, 1996; Snow et al, 1995; Snow et al, 1997; Snow et al, 1999; Tobin, 2005; Tobin et al, 2006), recombinant expression of the variable as well as constant region genes is required for whole antibodies. However, most well established methods of producing recombinant antibody at levels sufficient for biological assay require time- and labour- intensive methodologies (Bebbington et al, 1992; Filpula, 2007; Jain et al, 2007; Jones et al, 2003; Maynard & Georgiou, 2000; Zafir-Lavie et al, 2007). To address this, we have designed and constructed a set of cloning cassettes for antibody heavy, kappa and lambda chains in which restrictions sites flanking the variable and constant domain genes have been introduced by silent mutation (Figure 3.2). The restriction sites are compatible with all human constant gene isotypes and all human and murine germline variable genes, which allow exchange of either the variable or constant domain by restriction enzymes and ligase-dependent cloning. This means any conceivable human or mouse-human chimeric antibody may be expressed using this system.

To test the transient antibody expression system, I strove to produce cytokinergic IgEs and obtain the previously reported expression levels (Gan, 2008). pSGH and pSGK/pSGL vectors coding for the expression of 8 cytokinergic IgEs were co-transfected into HEK293E cells and supernatants harvested after 20 days to analyse antibody production by IgE specific ELISA. The expression levels of all tested IgEs, confirmed by ELISA, matched the previously reported expression levels, which enabled me to continue with the purification process and optimisation of the expression system

for better yields. The labour-intensive process and the high cost / low yield characteristics of HEK293E mammalian cell culture processes for antibody expression by large-scale transfection thus required optimisation of various production parameters to increase the volumetric antibody productivity.

An important parameter that affects antibody productivity is cell toxicity caused by the transfecting agent. Polyethylenimine (PEI) has previously been utilised for large-scale transfection into HEK293E cells (Pham et al, 2003) and shown to have low cell toxicity and rapid efficient uptake by cells (Godbey et al, 2000; Godbey et al, 1999a; Godbey et al, 1999b; Godbey et al, 1999c; Godbey et al, 1999d). To test the effectiveness of PEI in our expression system, an optimisation step with increasing PEI concentrations, whilst keeping the DNA concentration constant at 1 μg/ml, was carried out. Most favourable cell viability and maximum antibody expression level within 5 days incubation period was found to be the best at 4μg PEI : 1μg DNA ratio.

An important role of light-chain expression in Ig production has been shown (Baldi et al, 2005; Schlatter et al, 2005), suggesting a higher light-chain expression increases antibody production yield. To investigate and the role of light chain expression in our cloning system, optimisation of transfection conditions with HEK293E was carried out to determine the most effective light : heavy chain ratio. The ELISA analysis showed the optimum ratio of light : heavy chain DNA for the maximum antibody expression yield was found at 2:1, thus suggesting that double amount of light chain DNA contributed towards higher antibody production. This optimisation halved the DNA amount of the light chain previously used (4:1) to transfect cells, hence shortening the DNA production process. Furthermore, this result was consistent with the previously

observed requirement for light chain excess to render antibody folding and assembly more efficiently in transient transfections (Schlatter et al, 2005).

Previous studies (Pham et al, 2005) reported that addition of Tryptone N1 (a casein peptone, TN1) to the cell culture media 24 h post-transfection, led to a 2-fold increase in protein production after 5 days incubation. In addition, an Invitrogen product called Optimab, a monoclonal antibody production enhancer (consisting of alternate carbon sources, a monoclonal antibody production inducer, and essential nutrients been shown to be significantly depleted during the high-density culture phase), has been previously shown to increase antibody production. To maximise antibody yields, the culture media of transiently transfected HEK293E cells was supplemented independently with Tryptone N1 and Optimab 24h post-transfection. The ELISA analysis showed a 4-fold decrease in antibody expression level in the cultures supplemented with Tryptone N1 after 12 days incubation, suggesting the peptone depletion was not limiting antibody production yield in HEK293E cells. However, the Optimab supplementation represented a 2-fold increase in volumetric antibody productivity. Unfortunately, soon after the optimisation step was conducted, we discovered that Invitrogen discontinued Optimab. So far, no appropriate alternatives of Optimab have been found.

The transiently expressed IgEs antibodies were purified by affinity chromatography using IgG4-Fc-(sFcεRIα)$_2$ fusion protein (Shi et al, 1997). During purification of some IgE antibodies, an aggregation was observed, where certain combinations of IgE displayed aggregative tendencies. Therefore, the affinity column-purified IgEs were subjected to gel filtration/size-exclusion chromatography used for analysis and fractionation of monomeric antibodies and aggregated materials according to size (Hunt et al, 2005). Monomeric antibodies isolated from the size-exclusion chromatography

were concentrated and analysed by SDS-PAGE. Under non-reducing conditions, the purified IgEs appeared as single bands between 180 kDa and 250 kDa protein marker bands, corresponding to the expected ~190 kDa whole IgE molecule. Under reducing conditions, single bands were found at 25 kDa corresponding to the antibody light chains (25 kDa) and single bands found at 80 kDa possibly corresponding to glycosylated forms of the antibody heavy chains (~60 kDa). None of the purified antibodies showed significant contamination with other proteins, as judged by the absence of bands of molecular weight inconsistent with those expected for an IgE antibody polypeptide chains, indicating the affinity column purification and size-exclusion chromatography were effective in yielding pure monomeric IgEs.

Finally, to assess the biological functionality of the transiently expressed and affinity-purified cytokinergic IgE antibodies, I performed flow cytometric experiments, where RBL-SX38 cells expressing both human and rat FcεRI (Dibbern et al, 2003) were incubated with the IgEs and subsequently stained with anti-human IgE FITC. The flow cytometry analysis showed a clear right shift of fluorescence intensity, indicating the functional receptor-binding activity of the recombinant IgE antibodies.

Having successfully expressed, purified and characterised the previously cloned cytokinergic IgEs, I strove to demonstrate the versatility of our transient expression system to swap variable regions and its capacity to make fully functional antibodies, by cloning, expressing and characterising a High Molecular Weight Melanoma-Associated Antigen (HMW-MAA) specific IgE antibody. The existing V genes from pSGH and pSGK vectors were substituted with those specific for an HMW-MAA, utilising the BssHII and NheI restriction sites in the heavy chain cassette and SacII and BsiWI in the light chain cassette (Figure 3.11). The sequencing confirmation of the newly generated

pSGH-MAA and pSGK-MAA vectors demonstrated an effective exchange of the variable genes within pSG vectors using restriction enzymes and ligase-dependent cloning. Following successful PEI transfection of HEK293E cells with vectors coding for the expression of HMW-MAA specific IgE, the antibody expression level as determined by ELISA, was shown to be 0.7mg/L after 5 days incubation period. Antigen specificity and receptor-binding activity of the expressed HMW-MAA IgE antibody were analysed by flow cytometry The flow cytometric analysis of A375 melanoma cells incubated with the HMW-MAA specific IgE antibody, showed a clear right shift of fluorescence intensity, indicating preserved specific binding to native HMW-MAA as presented on the cell surface of A375 cells. No binding above background was detected to primary human melanocytes normally present in the skin of healthy patients. Furthermore, the IgE bound to human FcεR1 receptor, expressed on RBL SX38 mast cells (Dibbern et al, 2003), further demonstrating functional receptor-binding activity of the recombinant antibody.

To summarise, we have designed and produced a set of antibody cloning cassettes in which multiple cloning sites have been incorporated into the immunoglobulin coding sequence, enabling exchange of the variable and constant antibody genes by restriction enzymes and ligase-dependent cloning. This facilitates the expression of human or murine V genes of any human (or mouse-human chimeric) antibody isotype. The transient antibody expression system is capable of delivering 0.5-4mg/L antibodies, sufficient for antibody characterisation, produced in four weeks, from cloning of the V region through to harvesting of transfected cell supernatants, with preserved antigen binding and receptor-binding activities. This is particularly advantageous for characterisation of large libraries of V genes as a large panel of recombinant antibodies may be generated in a relatively short period of time compared to other available

antibody expression methods (Bebbington et al, 1992; Filpula, 2007; Jain et al, 2007; Jones et al, 2003; Maynard & Georgiou, 2000; Zafir-Lavie et al, 2007). Using the optimised protocol to clone and express HMW-MAA specific IgE antibody, an expression level of 0.7mg/L was detected 5 days after transient transfection. Thus, based on HMW-MAA IgE expression and expression levels of other IgEs expressed by the transient expression system, an estimated average production at 2mg/L of HMW-MAA specific IgE can be achieved in four weeks. To achieve this level, we estimate that four 500cm^2 triple layered flasks with adherent seeded cells will have to be transfected for a final volume of 1L. We planned to use HMW-MAA specific mAb as a control for our antibody discovery program, as well as for comparative functional studies between different isotypes in animal models. On an average, 10 mg of antibody is required to conduct one set of studies in a mouse model. Using our transient transfection system, transfection of 20 triple layered flasks would be required for 10 mg antibody production. One of the drawbacks of the pTT3 vector is that the backbone lacks a selection marker for generation of stable cell lines and thus contributes for the labour intensive procedure of repeating transfections. Although, the transient antibody expression system described in this chapter is capable of producing sufficient material for characterisations of multiple antibodies, it is unable to support animal model experiments in its current state. The modification of the transient antibody expression system to a stable antibody expression system, capable of producing over a 100mg/L antibody in four weeks has been described in my next chapter.

4 STABLE ANTIBODY EXPRESSION SYSTEM

4.1 Introduction

Over the last three decades, recombinant monoclonal antibodies (mAbs) have become a key tool for basic research, diagnosis and treatment of human diseases. The increasing demand for therapeutic antibodies has resulted in a significant improvement in antibody production systems, allowing biopharmaceutical communities to reach grams per liter expression levels. However, the lack of a suitable manufacturing platform, which ensures consistent antibody production, has always been one of the major impediments to the development of recombinant antibody material in academia.

Matched Ig heavy- and light-chain variable (V)-regions have been amplified from single B cell-derived cDNA encoding antibodies of the desired specificity (Koelsch et al, 2007; Smith et al, 2009; Tiller et al, 2008; Volkheimer et al, 2007; Wrammert et al, 2008) and linked to the desired Ig constant (C) region cDNA (Braren et al, 2007; Madritsch et al, 2011). However, there are still limitations in cloning and expression of recombinant mAbs. For example, co-transfection of separate vectors coding for either the heavy- or light-chain cDNA (Boel et al, 2000; Furtado et al, 2002; Koelsch et al, 2007; Madritsch et al, 2011; Tiller et al, 2008) might lead to low antibody expression, since the site of integration has a major effect on the level of expression of transfected genes (Wurm, 2004). Moreover, these antibody cloning systems rely on restriction enzyme- and ligase-dependent cloning procedures, whilst the restriction sites used in the vectors may not be compatible with all human constant gene isotypes. Our transient antibody expression system has overcome this limitation by incorporating restriction sites, compatible with all human constant gene isotypes and all human and murine germline variable genes, which allow exchange of either the variable or constant

domain by restriction enzyme- and ligase-dependent cloning. This facilitated the expression of human or murine V genes of any human (or mouse-human chimeric) antibody isotype, with preserved antigen binding and receptor-binding activities. Although the system is useful for the production of a large number of proteins in a short time, the lack of an antibiotic-based selection step limits expression yields and requires a costly and labour intensive scale-up process (Figure 4.1).

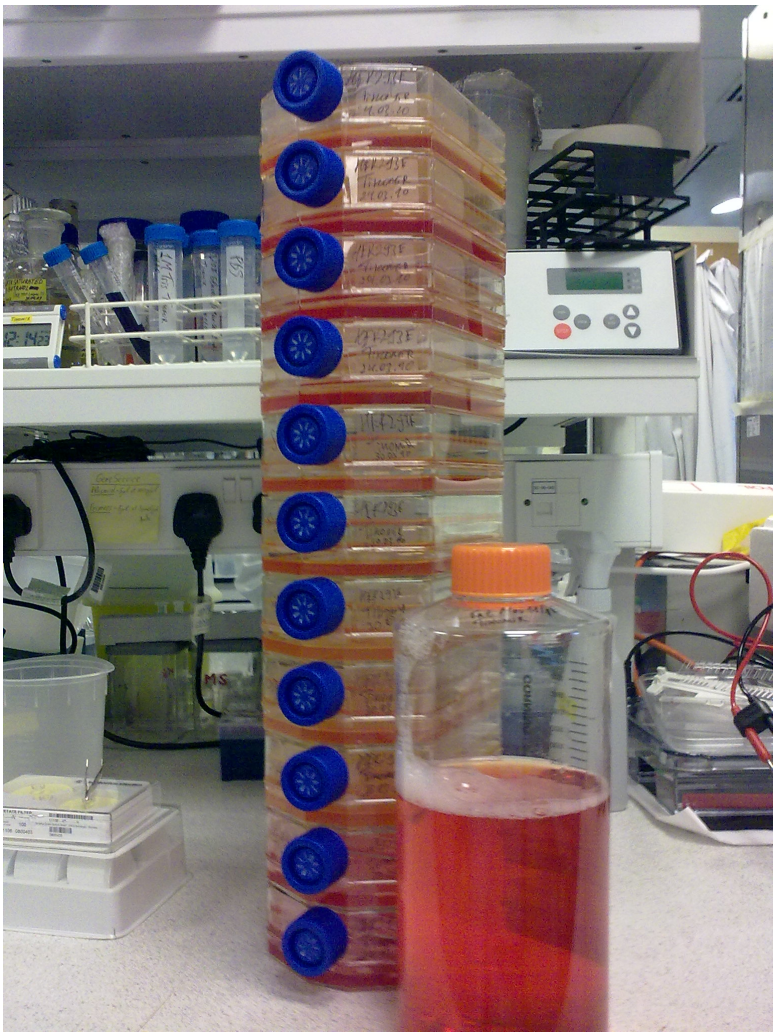


Figure 4.1: Triple layer flasks for transfections and transient expression of antibodies. Time-consuming large volume transfections in triple layer flasks are required for large-scale production of antibody using the transient expression system.

Different vectors hosting a dual antibody expression cassette have been exploited and shown to produce higher antibody expression levels (Braren et al, 2007; Hecker et al, 2011; Wiberg et al, 2006). However, they rely on restriction enzyme- and ligation-dependent cloning methods, which are time-consuming, prevent high-throughput analysis of antibody candidates and sometimes impractical due to the lack of compatible restriction sites. For example, swapping of variable genes within pSGH vector is possible due to unique *BssHII* and *NheI* restriction sites in the heavy chain cassette. However, the *BssHII* restriction site was present within the CDR3 region of clone G7 (Figure 4.2), the first antibody derived from our antibody discovery program (described in detail in chapter 6). This required a complex, labour-intensive cloning procedure (Figure 4.3).

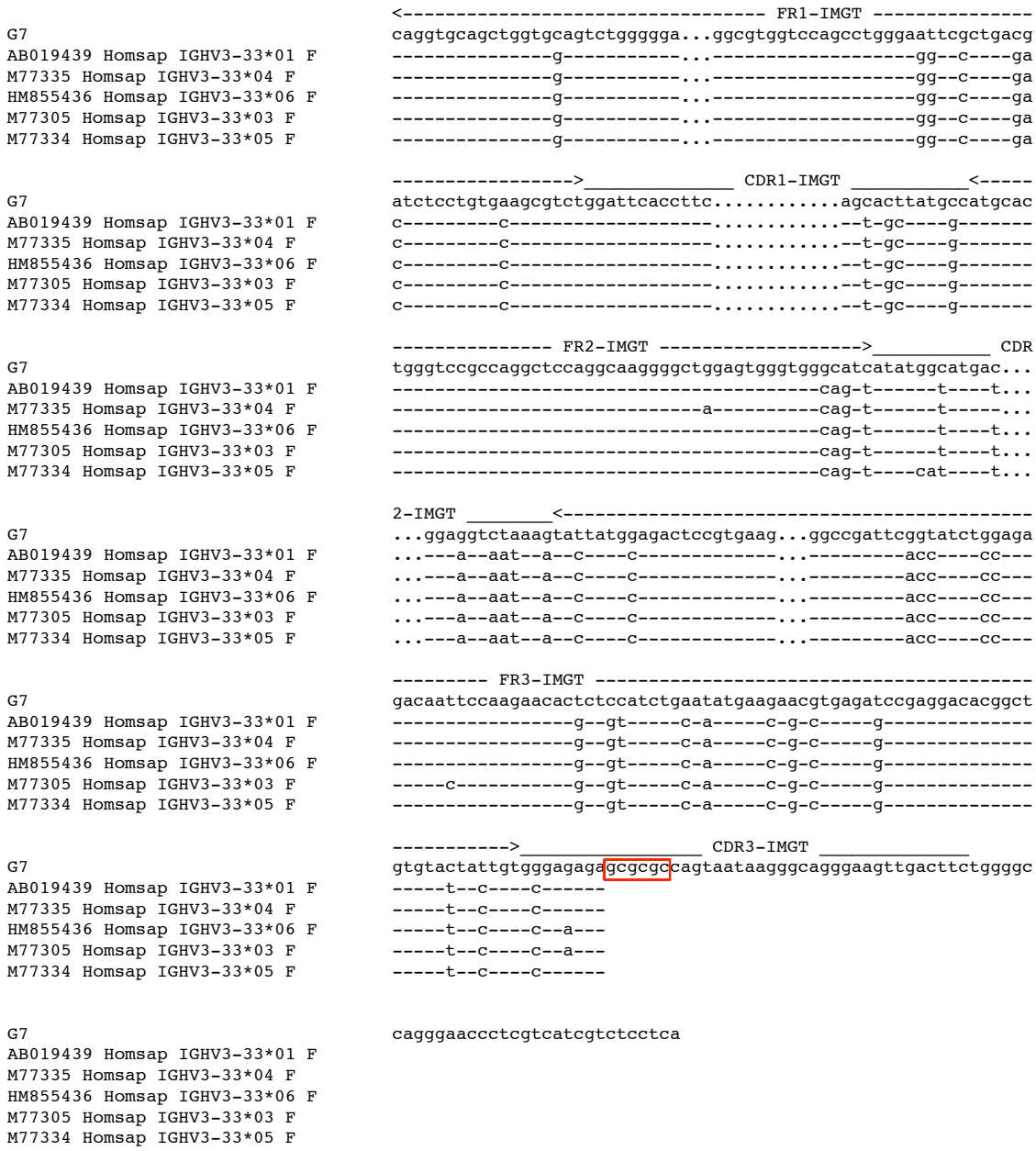


Figure 4.2: Schematic representation of restriction site within CDR3. Restriction site GCGCGC (in red box) present in the CDR3 of clone G7 is recognised by restriction endonuclease *BssHII*, an enzyme required for variable gene swapping in pSG vectors.

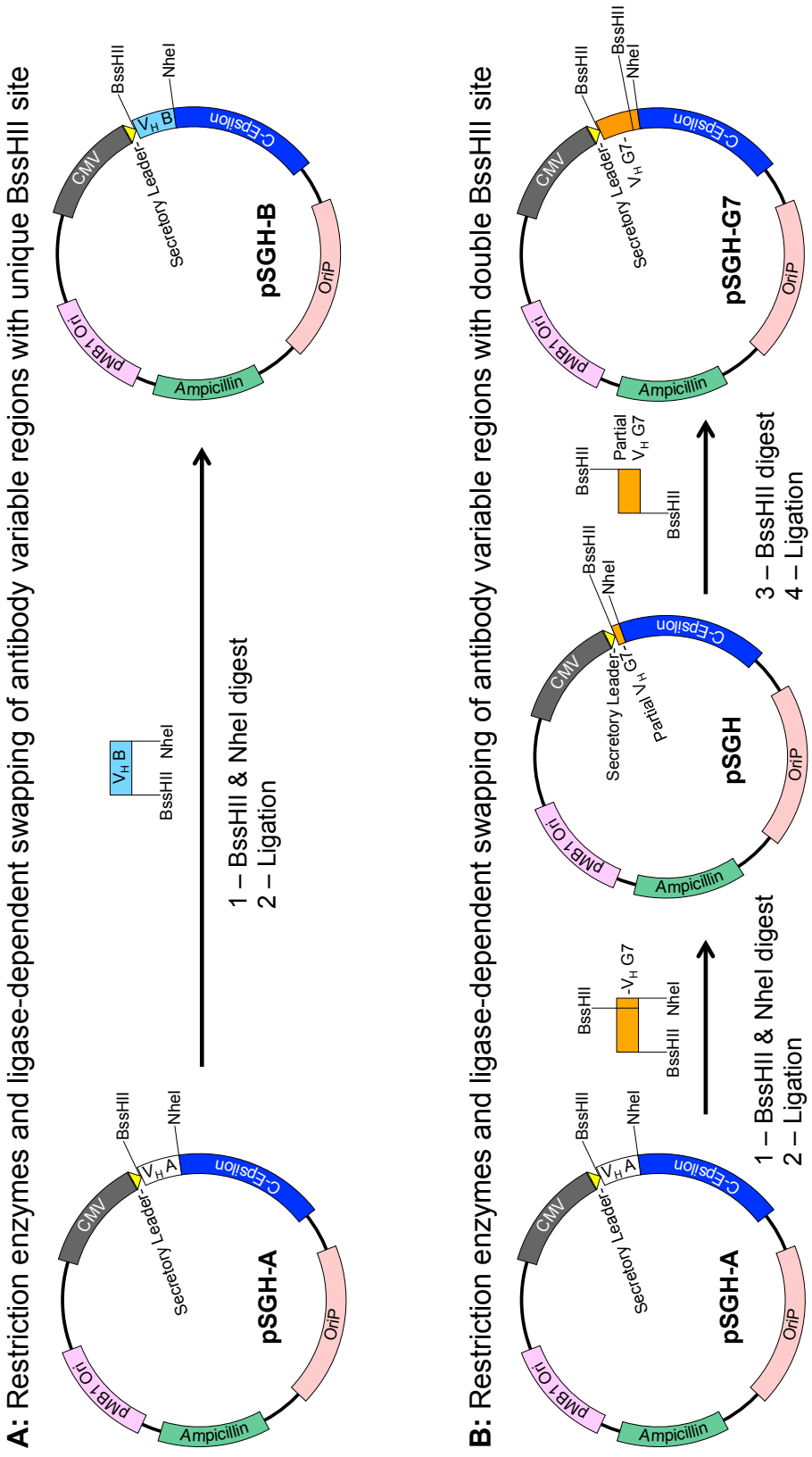


Figure 4.3: Schematic representation of restriction enzymes used for antibody cloning. (A) Swapping antibody variable regions by unique restriction enzymes. (B) Swapping antibody variable regions by restriction enzymes cutting within the variable region.

An alternative to conventional restriction enzyme- and ligation-dependent cloning methods is overlapping of DNA fragments for assembling antibody heavy- and light-chain cDNAs in expression vectors (Liao et al, 2009). An inherent difficulty of this approach is an additional PCR reaction relying on a specific annealing temperature of the overlapping fragments by means of a gradient PCR step.

I sought to generate an improved flexible system for the expression of antibodies with any desired specificity. My specific aims were as follows:

i) to minimise the time required to clone antibody heavy and light chains into a single mammalian expression vector and

ii) stable transfection of a suitable cell line that allows a scalable production process, which can deliver sufficient material for pre-clinical studies.

In order to demonstrate the system's versatility and capacity to make fully functional antibodies and to fulfil current limitations, I have developed a dual antibody expression cassette that facilitated the seamless exchange of variable and constant domain genes to produce HMW-MAA specific IgE and IgG4 antibody isotypes as a proof-of-principle. The choice of these antibodies was dictated by the search for therapeutic antibodies for the immunotherapy of melanoma and the availability of functional assays for the IgE and IgG4 isotypes in our laboratory. In this chapter I have described an easy and efficient method for antibody cloning and production based on established techniques, which is novel in their combination and application.

4.2 Methods

4.2.1 Generation of a dual antibody expression cassette

In order to integrate the heavy chain expression cassette into multiple cloning site 2 (MCS2), pVITRO1 was linearised (Figure 4.5 1A) using primers MCS2_F and MCS2_R (Table 2.2). In parallel, the heavy chain expression cassette from a previously constructed vector (pSGH-G7) was amplified using primers HC_F and HC_R (Table 2.2), in which the 5′-ends are complementary to the MCS2 of pVITRO1 and the 3′-ends complementary to the V_H1-02 secretary leader and Cε terminus respectively (Figure 4.5 1B).

The linearisation of the vector and amplification of the heavy chain expression cassette was performed using Phusion™ Flash High-Fidelity PCR Master Mix (Finnzymes) according to the manufacturer's instructions, except the final extension step was omitted. The unpurified PCR products (heavy chain DNA and linearised pVITRO) were mixed 1:1 (v/v) as previously described (Klock et al, 2008), and 2 µl of the mixture was transformed into XL1-Blue competent cells (Stratagene) according to the manufacturer's instructions. Transformed cells were plated on LB petri dishes supplemented with 50 µg/ml Hygromycin B (Invitrogen). There was no risk of background template contamination at this stage, as the pSG vectors have ampicillin resistance only. Single colonies were picked and inoculated in 5 ml LB broth (Invitrogen) supplemented with 50 µg/ml Hygromycin B. After overnight culture at 37°C with shaking at 200rpm, bacteria were harvested and plasmid DNA purified with a Wizard Plus SV Minipreps DNA Purification System (Promega). Purified plasmid DNA was then sequenced with primers pVitro1F and pVitro1R (Table 2.2) to confirm integration of the heavy chain expression cassette within MCS2. The newly generated vector was named pVitro-IgH. Moreover, this confirmed that the PIPE cloning method

is extremely quick and cost effective, which encouraged me to continue with the construction of the dual antibody expression cassette in pVITRO1.

The light chain expression cassette was then integrated into MCS1 within pVitro-IgH. First the vector was linearised using primers MCS1_F and MCS1_R (Table 2.2). The light chain expression cassette was amplified from a previously constructed pSGK-G7 vector using primers LC_F and LC_R (Table 2.2), in which the 5′-ends are complementary to the MCS1 and 3′-ends complementary to the V_K A26 secretary leader and Cκ terminus respectively. Vector construction was performed as described for the heavy chain. Sequencing confirmed the integration of the light chain expression cassette within MCS1 thus validating the generation of a novel expression vector, pVitroG7-IgE, coding for the expression of G7 IgE antibody.

4.2.2 Swapping variable regions

pVitroG7-IgE vector was linearised by two sets of primer pairs flanking G7 V_K and V_H; Linear_Kfwd/Linear_Hrev and Linear_Hfwd/Linear_Krev (Table 2.2), in two independent PCR reactions. The two resulting vector fragments were treated with the endonuclease enzyme *Dpn I* (NEB) which destroys the *E.Coli*-derived vector template (Figure 4.7) thus reducing background contamination from pVitroG7-IgE vector.

Simultaneously, the HMW-MAA specific V_H and V_K were amplified by primer pair MAAH_Fwd and MAAH_Rev and primer pair MAAK_Fwd and MAAK_Rev (Table 2.2) respectively in two independent PCR reactions, for generation of vector fragment terminal end-homology (Figure 4.7). The *Dpn I* treated vector fragments were mixed with unpurified HMW-MAA specific V_H and V_K in 1:1:1:1 (v/v) ratio and 2 µl from the

mixture transformed into XL1-Blue competent cells as described previously (Section 4.2).

4.2.3 Swapping constant regions

To exchange Cε with Cγ4 within pVitroMAA-IgE construct, the vector was linearised, using primer pairs flanking the Cε region (pAn_Fwd and VH_Rev, Table 2.2) and subsequently treated by *Dpn I* (NEB) (Figure 4.9). The human Cγ4 region was amplified from an existing pSG vector (James et al, 2012) using primer pair Cg4_Fwd and Cg4_Rev (Table 2.2), for generation of vector fragment terminal end-homology. The *Dpn I* treated linearised vector and unpurified Cγ4 PCR product were mixed in 1:1 (v/v) ratio, followed by 30 minutes incubation at 60°C and 2 µl from the mixture transformed into XL1-Blue competent cells. The newly generated vector was named pVitroMAA-IgG4, coding for the expression of chimeric HMW-MAA specific IgG4 antibody (Figure 4.9).

4.2.4 Stable expression of antibodies

293-F were cultured at a density of 1 - 3x10^6cells/ml in FreeStyle™ 293-F Expression Medium (Life Technologies) in sterile 125 ml Erlenmeyer flasks (Sigma), rotating at 135 rpm on orbital shaker platform. For transfection, 3x10^7 cells were transfected with 40 µg of pVitroMAA-IgE or pVitroMAA-IgG4 DNA, coding for the expression of the HMW-MAA specific IgE and IgG4 respectively, using FreeStyle™ MAX transfection reagent according to manufacturer's instructions. For the generation of stable cell lines, 50 µg/ml Hygromycin B was added to the media 24 h post-transfection, and the cells were kept under selection for 2 weeks at 1-3x10^6 viable cells/ml. 1 ml samples were collected every 48 h for antibody quantification by anti-human IgE ELISA as described

previously (McCloskey et al, 2007). The Hygromycin-selected cells expressing the HMW-MAA specific IgG4 were expanded into 1L spinner and the supernatants harvested after two weeks. The antibody expression level was determined by anti-human IgG4 ELISA as described previously (James et al, 2012).

4.2.5 Purification of IgG

Cell supernatants were harvested and centrifuged at 1000 x g for 15 minutes to remove cell debris. Supernatants were passed over 0.45μm filters (Sartorius) and stored at 4°C with 0.1% sodium azide (Sigma) until purification. The HMW-MAA-specific IgG4 isotype was purified by affinity chromatography with a 5 ml HiTrap Protein-G HP column (GE Healthcare) using an ÄKTA Prime system (Amersham, Uppsala Sweden). The column was equilibrated with 10 Column Volumes (CV) of washing buffer (PBS, pH 7.0). Filtered supernatant was loaded onto the column at a flow rate of 2 ml/min and the column washed with 10 CV washing buffer. The antibody was eluted with 0.2M Glycine, pH 2.5 and 2.5 ml fractions were collected into tubes containing 0.5 ml 1M Tris–HCl pH 8.6 for neutralization.

4.2.6 Flow Cytometry

Recombinant HMW-MAA-specific IgE and IgG4 were incubated with HMW-MAA+ (A375 melanoma cells) and HMW-MAA- (primary human melanocytes) cells for 30 min at 4^{o}C. Cells were washed twice in PBS supplemented with 5% normal goat serum (FACS buffer). Cells were then incubated with 10 μg/ml anti-human IgE-FITC (Jackson ImmunoResearch) or IgG-FITC (Jackson ImmunoResearch) for 30 min at 4^{o}C and washed with FACS buffer prior to acquisition and analysis on a FACSCanto^{TM} flow cytometer (BD Biosciences).

4.3 Results

4.3.1 Design and construction of a versatile dual antibody expression cassette

The first antibody variable regions (clone G7) derived from our antibody discovery program (described in detail in chapter 6) were cloned utilising *BssHII* and *NheI* restriction sites and a ligase-dependent cloning procedure (Figure 4.2). However, this was a labour- and time-intensive proves, hence, I strove to improve flexibility and reduce the cloning time of this method, by adapting the Polymerase Incomplete Primer Extension (PIPE) method (Klock et al, 2008). I also aimed to maximise expression yields by designing and constructing a dual antibody expression cassette using the pVITRO1 mammalian expression vector (InvivoGen). This enables stable transfection in a mammalian cell line and an easily scalable production process, which can deliver sufficient material for animal model experiments. The pVITRO1 mammalian expression vector (Figure 4.4), has multiple advantages over pSG vectors, making it much more suitable for expression of antibodies. Firstly, pVITRO contains a dual expression cassette for simultaneous expression of both heavy and light chain genes, thus eliminating the requirement for co-transfection of two vectors and halving the time and effort needed for DNA preparation and transfection. Second, efficient recombinant antibody production is dependent on the ratio of heavy to light chain gene expression (Schlatter et al, 2005). I therefore placed the heavy chain expression cassette under the control of the Simian Virus 40 (SV40) enhancer and the light chain expression cassette under the control of human cytomegalovirus (HCMV) enhancer. HCMV is several fold more active than the SV40 enhancer so maintains the optimal ratio of Heavy to Light chain gene expression. Finally, pVITRO contains the hph gene, part of the vector backbone, which confers resistance to Hygromycin B in both *E. coli* and mammalian cells, allowing a selection step and generation of stable cell lines.

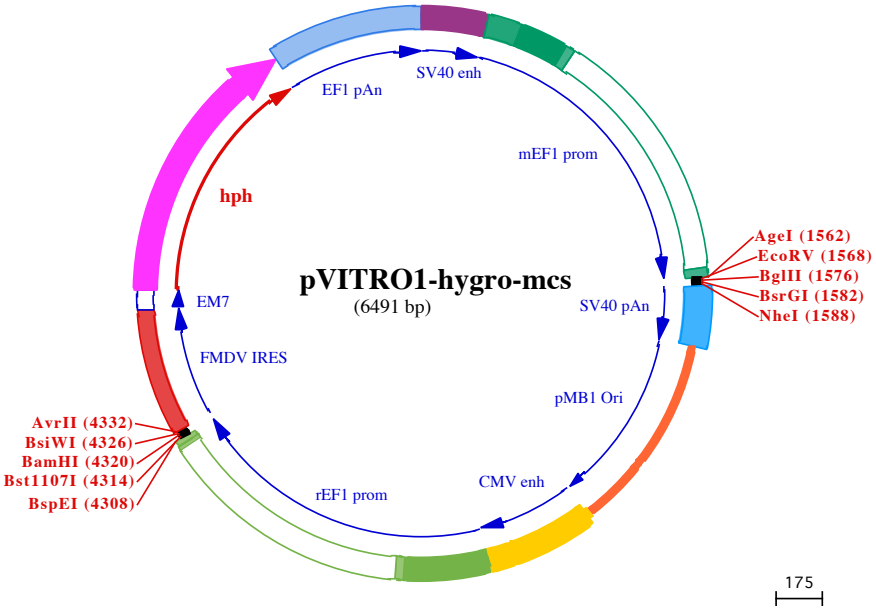


Figure 4.4: Schematic representation of the pVITRO1 vector. pVITRO1 mammalian expression vector (InvivoGen) used for construction of a dual antibody expression cassette with Hygromycin selection marker for generation of stable cell lines

Cloning the heavy and light chain expression cassettes into a large vector negatively affects the swapping of variable or constant genes due to the lack of compatible restriction sites, reinforcing the demand for an alternative to the conventional restriction enzyme- and ligation-dependent cloning methods. The PIPE cloning method has been shown to be extremely quick, cost effective, and highly efficient, capable of supporting the high-throughput cloning of thousands of genes in parallel (Klock et al, 2008). This method eliminates the use of restriction enzymes and ligation, and thus the incorporation of restriction sites, which could sometimes encode extra, unwanted residues into expressed proteins (Hartley et al, 2000). The PIPE method relies on the inefficiency of the amplification process in the final cycles of a PCR reaction, possibly due to the decreasing availability of dNTPs. This incomplete 5′-3′ primer extension results in a mixture of products with variably single-stranded 5′-ends (Olsen & Eckstein, 1989). The single-stranded 5′-ends are homologous to the 5′ region of the primers. The

cloning method represents a simple two-step protocol with complete design flexibility. In the first step, a set of vector-specific primers are used for PCR vector linearisation and another set of primers with 5'-vector-end overlapping sequences are used for insert amplification, generating incomplete extension products by PIPE. In the second step, the single-stranded overlapping sequences of the incomplete extension products are mixed with complementary strands which then anneal and assemble as a complete vector (Klock et al, 2008). I adapted the PIPE method for antibody cloning and construction of the dual antibody expression cassette in pVITRO1. The basic rules for designing primer pairs when swapping variable (V) or constant (C) antibody regions by PIPE are illustrated in details in Figure 4.5. Integrating the heavy and light chain expression cassettes into pVITRO1 (Figure 4.6), completed the construction of a dual antibody expression cassette within a mammalian expression vector, using a restriction enzyme- and ligation-independent cloning method – PIPE.

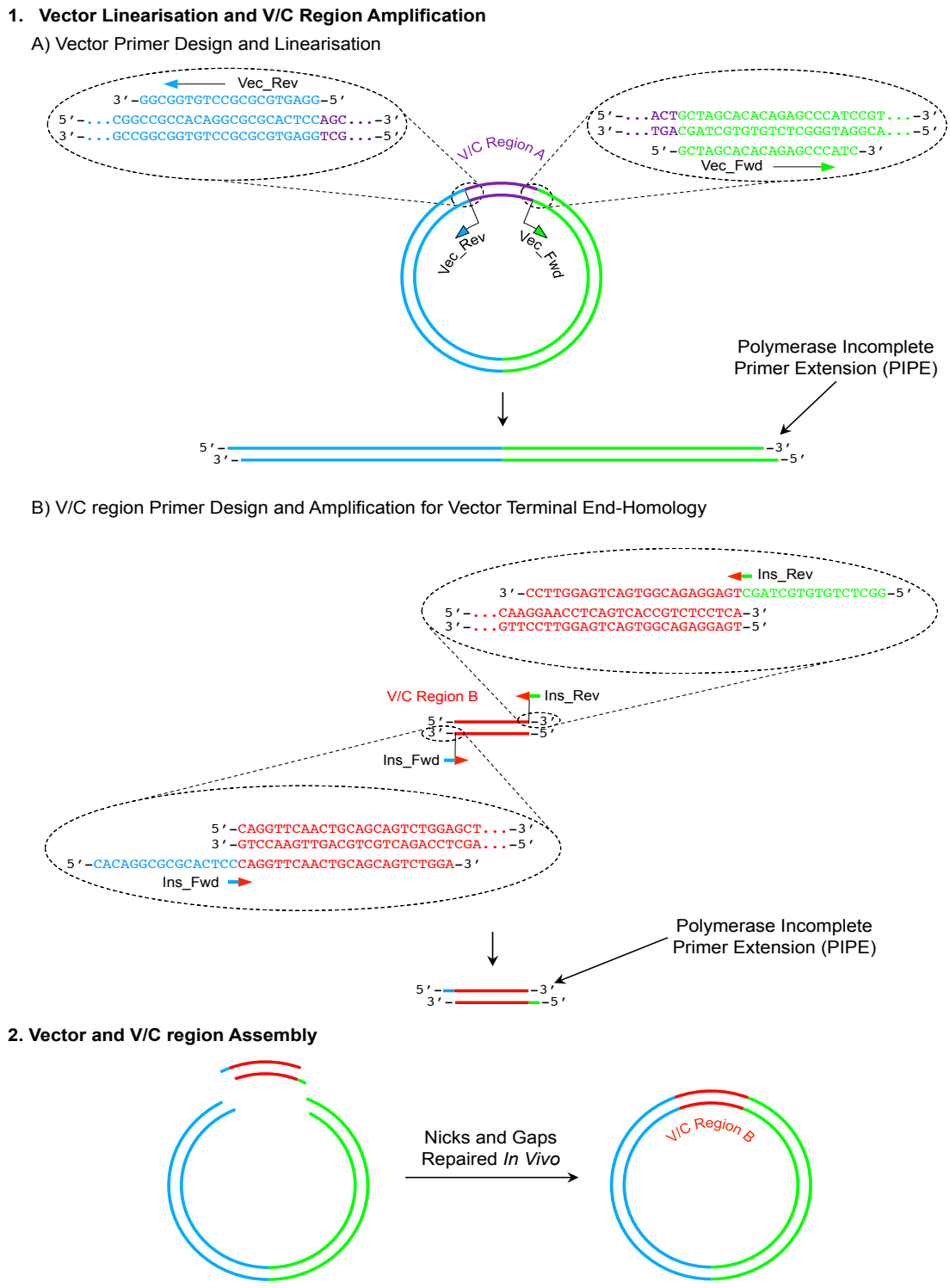


Figure 4.5: Schematic representation of primer design for PIPE cloning. 1 A) A set of primers flanking the existent V/C antibody region are designed for vector linearisation. 1 B) Simultaneously, the desired V/C antibody region is amplified by another set of 3' V/C region specific, 5' vector-end homologous primers. 2) The incomplete extension products anneal directionally across the complementary sequences encoded in the primers and nicks and gaps are repaired *in vivo* after transformation.

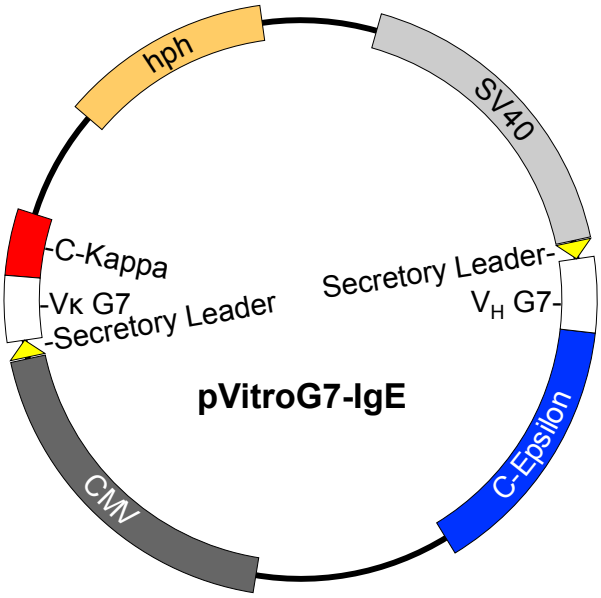


Figure 4.6: Schematic representation of the dual antibody expression cassette. pVITRO1 mammalian expression vector with heavy chain expression cassette integrated under the action of the Simian Virus 40 (SV40) enhancer and light chain expression cassette under human cytomegalovirus (CMV) enhancer, several fold more active than the SV40 enhancer, coding for the expression of G7-IgE antibody.

4.3.2 Swapping variable regions by PIPE

Having successfully utilised the PIPE cloning method for construction of the dual antibody expression cassette, I next optimised the procedure for swapping the specificity of the antibody in a single day. This process requires exchanging V_H and V_K regions in pVitroG7-IgE, without affecting the Cκ and Cε. To achieve this, four primer pairs were designed. The first two primer pairs, flanking the existing V_H and V_K, were used for double fragment linearisation of the pVitroG7-IgE vector. The second two primer pairs, with 5'-fragment-end overlapping sequences, were used for amplification of the incoming V_H and V_K. The primers were used to exchange the variable regions (V_H and V_K) from pVitroG7-IgE vector with a V_H and V_K directed against the human HMW-MAA (murine monoclonal antibody 225.28S,(Neri et al, 1996)) (Figure 4.7).

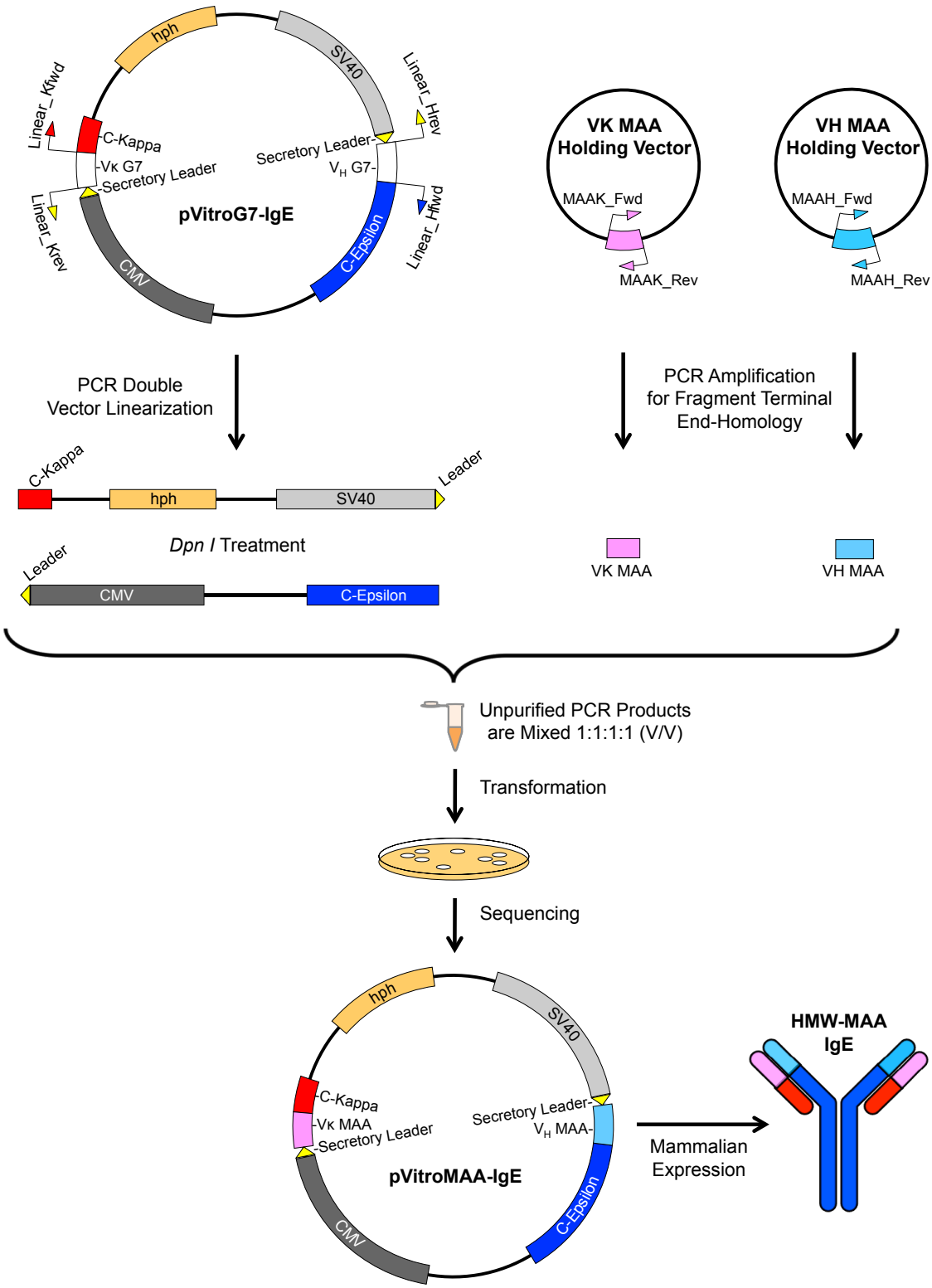


Figure 4.7: Schematic representation of variable genes swapping by PIPE. pVitroG7-IgE vector is linearized by V_H and V_K flanking primer pairs in two independent PCR reactions, resulting in two vector fragments subsequently treated by *Dpn I*. Simultaneously, the HMW-MAA specific V_H and V_K are amplified for generation of vector fragment terminal end-homology. The vector fragments are mixed with unpurified HMW-MAA specific V_H and V_K. The single-stranded DNA fragments anneal directionally across the complementary sequences and nicks and gaps are repaired *in vivo* after transformation, generating pVitroMAA-IgE expression vector.

DNA sequencing of a single colony confirmed the correct assembly of the vector fragments with the HMW-MAA specific V_H and V_K by the PIPE cloning reaction in pVitroMAA-IgE, coding for the expression of chimeric HMW-MAA specific IgE (Figure 4.7). This cloning experiment has proved the exchange of the specificity of an antibody by PIPE cloning is possible in a single day. This is very advantageous for the high-throughput screening of antibody candidates derived from our antibody discovery program (described in details in chapter 6).

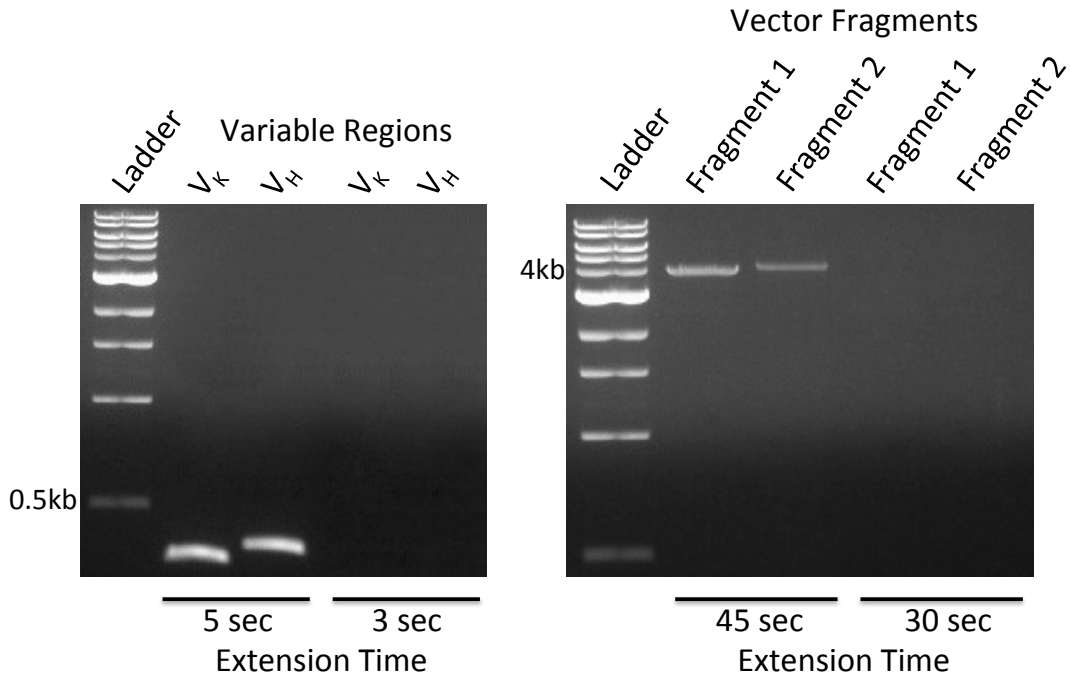
4.3.3 PIPE cloning optimisation

The use of PIPE cloning for exchange of the variable antibody regions in the dual antibody expression vector is both time- and cost-efficient. However, during the initial cloning experiments, low cloning efficiency was observed with only one or two colonies per plate. The PIPE method relies on the inefficiency of the polymerase to amplify polynucleotide chains in the final cycles of a PCR reaction, possibly due to the decreasing amount of dNTPs available and more template copies that remain to be synthesised (Olsen & Eckstein, 1989). Based on these observations, I aimed to increase cloning efficiency by increasing the number of single-stranded 5′-ends to provide more complementary strands to anneal. This was done by decreasing the dNTPs and template concentration in the PCR reaction, as well as minimising the extension time needed by the polymerase to synthesise the polynucleotide chain.

During initial PIPE cloning experiments, Phusion™ Flash High-Fidelity PCR Master Mix (Finnzymes) was used according to the manufacturer's recommendations, except the final extension step was omitted. In order to optimise the reaction for the purposes of PIPE cloning I compared template concentration, initial denaturation time as well as duration of the denaturation, annealing and extension within the PCR cycles. I found

that 1 ng template (versus 10 ng) was sufficient to obtain a PCR product (data not shown). I also found that a shortened initial denaturation step (30 seconds versus 2 minutes) and shortened denaturation, annealing and extension times within the cycles (10s, 15s and 5-55s vs 30s, 30s, 15s respectively) led to an improved PCR (Figure 4.8). Next, to test the effect of temperature on transformation efficiency, variables and vector fragments generated with optimised conditions as described above, were mixed with 1:1:1:1 (v/v) ratio and incubated for 30 minutes at RT, 50°C, 55°C, 60°C, 65°C, 70°C and 75°C before transformation into XL1-Blue competent cells. This experiment showed the highest cloning efficiency was present when the reaction was incubated for 30 minutes at 60°C, representing 20 colonies per plate.

A) Initial Extension Time Optimisation



B) Optimised Extension Time

Cycle step	Temp.	Time	Cycles
Initial denaturation	98°C	30 s	1
Denaturation Annealing Extension	98°C 60°C 72°C	10 s 15 s 5/55 s	30
	4°C	hold	1

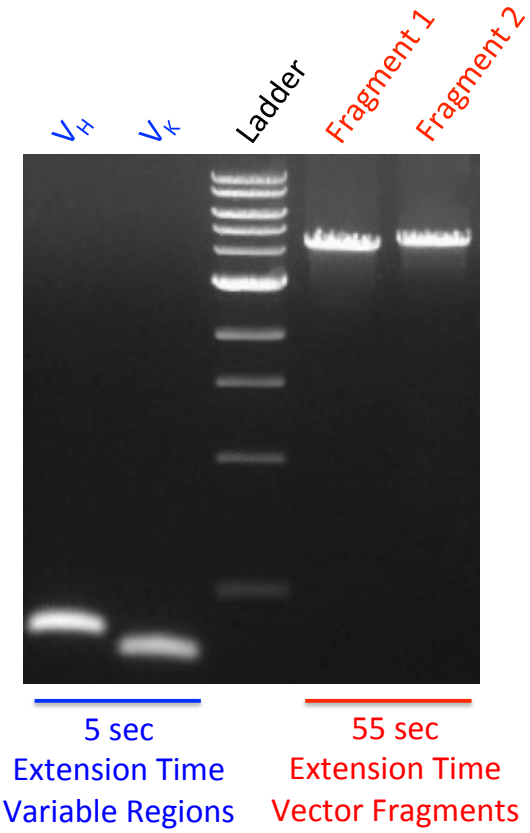


Figure 4.8: Extension time optimisation. Extension time optimisation of Phusion™ Flash polymerase for generation of incomplete extension products required for PIPE cloning, using HMW-MAA specific variables and vector fragments.

4.3.4 Swapping Constant Regions by PIPE

To take advantage of the varied effector functions conferred by different isotypes, it is often advantageous to compare two or more isotypes of immunoglobulin with the same specificity e.g to demonstrate differential tissue distributions between subclasses. Therefore to show that the pVITRO system is capable of exchanging isotypes and expressing functional antibodies, the C_H region of pVitroMAA-IgE was exchanged from Cε to Cγ4. These cloning experiments represent the system's simplicity to exchange variable- or constant-region antibody domains allowing cloning of different isotypes with any desired specificity. The HMW-MAA specific IgE and IgG4 antibody isotypes were then expressed in parallel and characterized in terms of their respective effector functions.

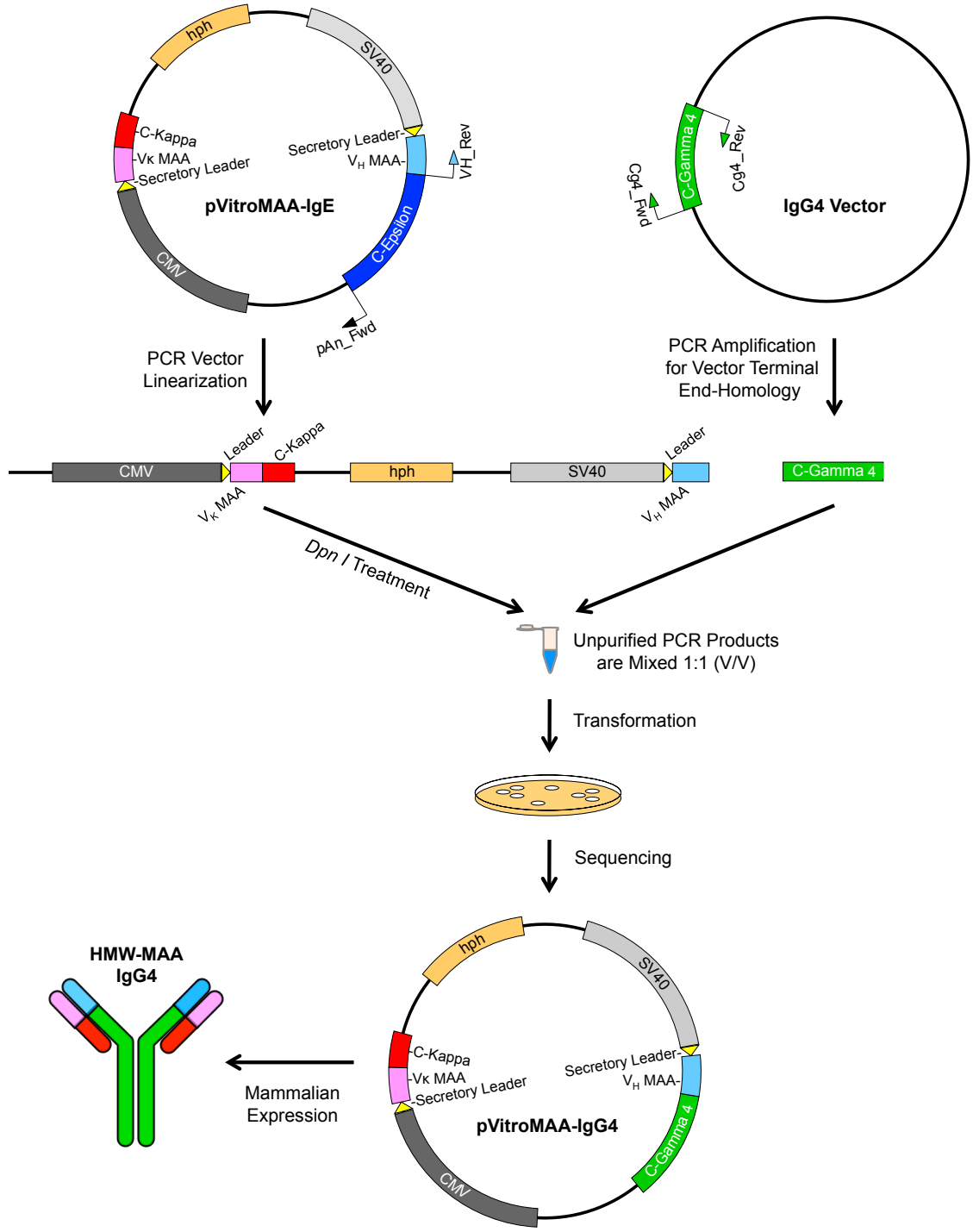


Figure 4.9: Schematic representation of isotype swapping cloning procedure by PIPE. pVitroMAA-IgE expression vector is PCR linearised by C-Epsilon region flanking primers and subsequently *Dpn I* treated. Simultaneously, C-Gamma 4 region is PCR amplified for generation of vector terminal end-homology. The unpurified *Dpn I* treated linearised vector is mixed 1:1 (v/v) with unpurified C-Gamma 4 PCR product. The single-stranded DNA fragments anneal directionally across the complementary sequences and nicks and gaps are repaired *in vivo* after transformation, generating pVitroMAA-IgG4 expression vector.

4.3.5 Stable expression and purification of recombinant antibodies

Having successfully adapted and optimised the PIPE cloning method for exchange of variable- or constant-region domains within the dual antibody expression cassette in pVITRO1, I then generated stable cell lines to support large-scale antibody production for subsequent use in animal model studies. The mammalian cell line, FreeStyle^{TM} 293-F cells (Life Technologies), is a suspension culture that has been adapted to grow in serum-free conditions. This makes it particularly suited for antibody production due to its easy scalability and for subsequent purification.

The FreeStyle^{TM} 293-F cells were transfected as previously described and Hygromycin-selected cells expressing HMW-MAA specific IgE were expanded into a 1L shaker flask (Sigma), 1L spinner bottle (Sigma) or 5L WAVE bioreactor (GE Healthcare) (Figure 4.10A)

To compare the final expression yields between the transient and stable antibody expression system, 293-F cells were transiently transfected with pSGH-MAA and pSGK-MAA vectors (Section 3.10), also coding for the expression of HMW-MAA specific IgE. Transiently transfected cells were cultured in IL shaker flasks and 1 ml samples were collected every 48 h, until day 21. At this point, as expected all of the cells were dead and the culture was therefore terminated. Antibody concentrations were determined by anti-human IgE ELISA.

Transient antibody expression produced a maximal 1 mg/L expression yield by day 13 (Figure 4.10B). In contrast, after 2 weeks selection, the stable cell lines expanded into 1L shaker flask, 1L spinner and 5L WAVE bioreactor and produced 10 mg/L, 15 mg/L and 25 mg/L of respective antibody expression levels at 30 days post-transfection (Figure 4.10B). Using a 5L WAVE bioreactor, 125 mg total amount of antibody was produced within 30 days. The ELISA analysis showed the HMW-MAA specific IgG4 antibody was expressed at 70 mg/L within 2 weeks using 1L spinner (Figure 4.11).

Shaker Flasks

Spinners

Wave Bioreactor

Figure 4.10A: Schematic representation of tissue culture vessels. The large-scale production of recombinant antibodies was conducted in shaker flasks (1L), spinners (1L) and Wave bioreactor (5L).

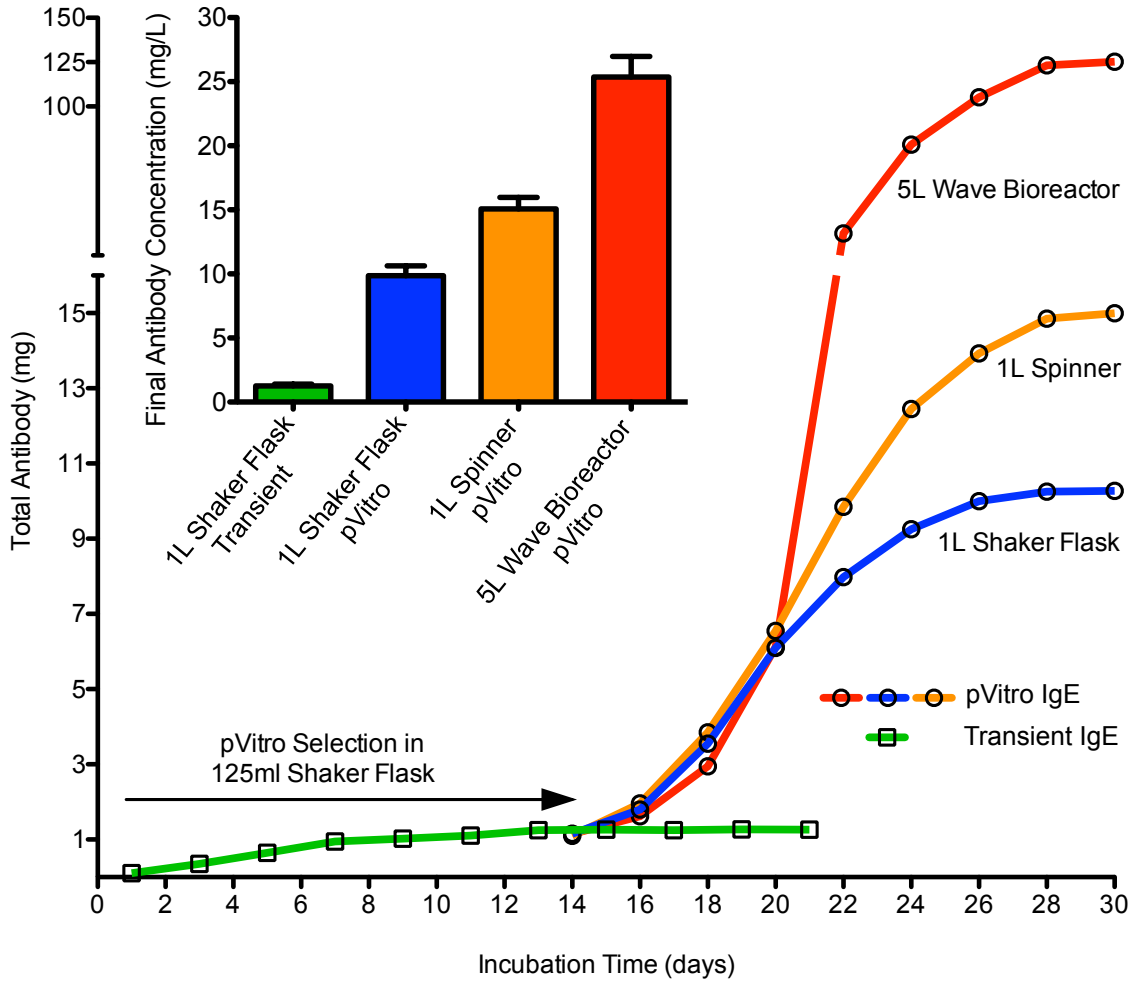


Figure 4.10B: Stable cell line generation and large-scale production of recombinant antibodies. IgE ELISA comparison of antibody expression yields. Sample supernatants from two 293-F cell cultures transfected with either two transient monocistronic vectors pSG (in squares) or pVitro bicistronic expression vector (in circles) encoding for the HMW-MAA specific IgE antibody at different time points. pSG transfected cell cultures (in green) lacking a selection marker terminate within 3 weeks post-transfection. pVitro transfected low volume cell cultures, undergoing a two-week Hygromycin selection for generation of stable cell lines, are scaled up to larger volume shaker flasks (in blue), spinners (in orange) or bioreactors (in red).The antibody concentration at mg/L was determined by reference to a standard curve and the results represent the mean of triplicate readings ± SD.

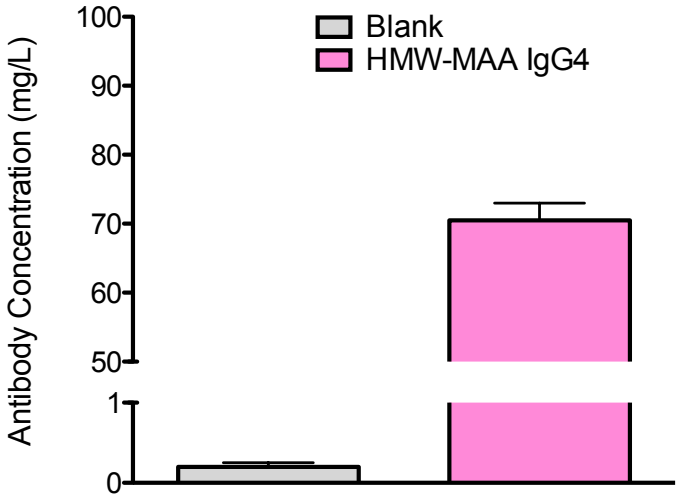


Figure 4.11: Stable expression of HMW-MAA specific IgG4. Anti-human IgG4 ELISA analysis of HMW-MAA specific IgG4 stable expression in 1L spinner. The antibody concentration at mg/L was determined by reference to a standard curve and the results represent the mean of triplicate readings ± SD.

The HMW-MAA-specific IgE isotype was purified by affinity chromatography using IgG4-Fc-(sFcεRIα)$_2$ fusion protein, as previously described (Methods 3.2). The HMW-MAA-specific IgG4 isotype was purified by affinity chromatography with a 5 ml HiTrap Protein-G HP column (GE Healthcare) using an ÄKTA Prime system (Amersham, Uppsala Sweden). The eluted IgG4 was present in fractions 2-7 with a shoulder elution profile characteristic for IgG elution (Figure 4.12).

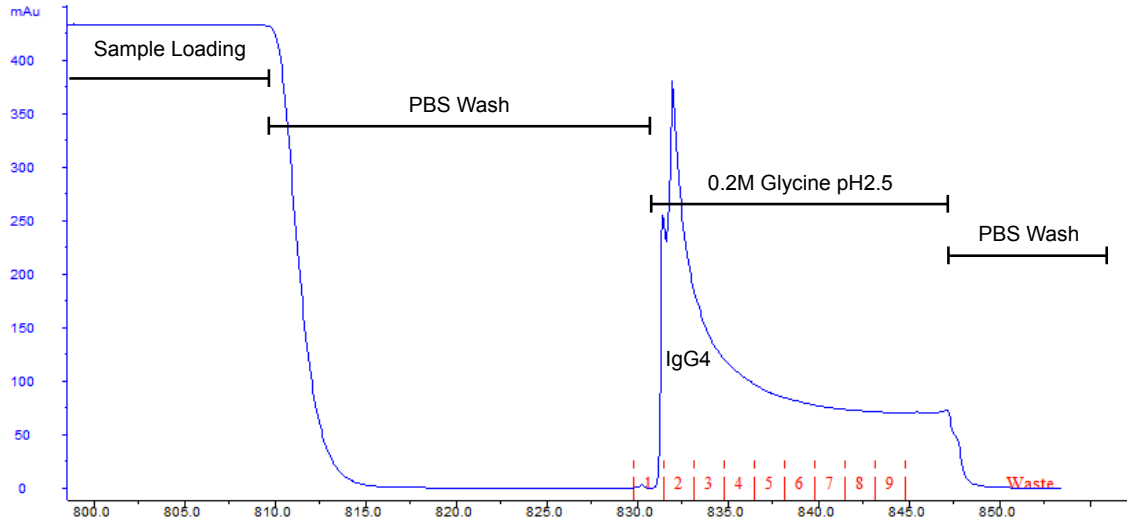


Figure 4.12: Affinity chromatography analysis of stably expressed IgG4. Elution profile from affinity purification of stably expressed IgG4 on a Protein G column by AKTA Prime. IgG4 is eluted by low pH in the 2-7 fractions.

4.3.6 Size-exclusion chromatography of affinity purified HMW-MAA IgE and IgG4.

Size-exclusion chromatography was used for biophysical analysis of the affinity column-purified HMW-MAA specific IgE and IgG4 and 2 isotype controls; MOv18 IgE is an antibody directed against an ovarian cancer antigen and entering Phase I clinical trials (Karagiannis et al, 2003) and a commercially available human myeloma IgG4 (Millipore) respectively, as previously described (Hunt et al, 2005). Gel filtration was performed on a Gilson HPLC system using a Superdex™ 200 10/300 GL column (Amersham, Uppsala Sweden) as previously described (Methods 3.2). The size-exclusion chromatography analysis showed no aggregation and confirmed the affinity column-purified product consists of monomeric antibodies (Figure 4.13).

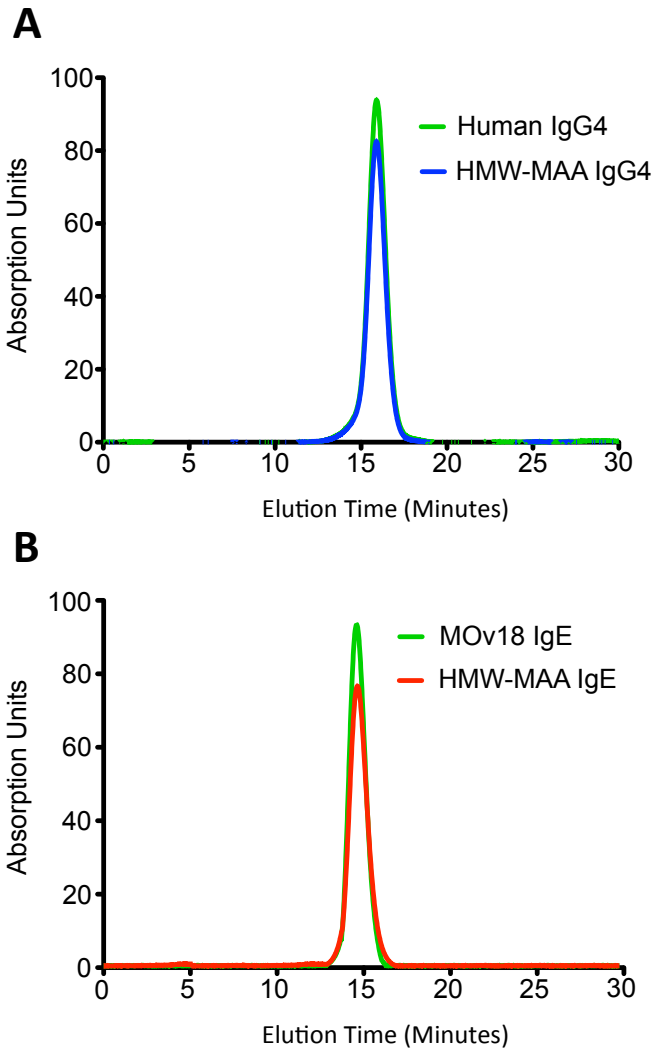


Figure 4.13: Size exclusion chromatography analysis of HMW-MAA specific IgG4 and IgE. Elution profiles of recombinantly expressed and affinity purified HMW-MAA specific IgG4 (A) and IgE (B) antibodies from Superdex™ 200 gel filtration. The antibody profiles correspond to isotype controls human myeloma IgG4 and MOv18 IgE respectively, and confirm the affinity purified products consist of monomeric antibodies.

4.3.7 SDS-PAGE analysis of affinity purified HMW-MAA IgE and IgG4

The size and purity of affinity column-purified antibodies was assessed by SDS-PAGE analysis. 2 µg each HMW-MAA specific IgE and IgG4 and isotype controls MOv18 IgE and human myeloma IgG4 (Millipore) were subjected to a gradient 5–12% polyacrylamide gel electrophoresis under non-reducing and 5–20% polyacrylamide gel under reducing conditions. Spectra™ Multicolor High Range Protein Ladder (Fermentas) was used to assess the molecular weight of the protein bands as visualised by Coomassie blue staining.

Under reducing conditions, protein bands corresponding to the heavy (52-72 kDa) and light chains were visible for both HMW-MAA specific IgG4 and the control human myeloma IgG4 (Figure 4.14B). Similarly, the HMW-MAA IgE and control MOv18 IgE were visible at 72-95 kDa, corresponding to the heavy chain with a lower band corresponding to the light chain. Under non-reducing conditions, molecular sizes were found to be in between 180-250 kDa for the heterotetrameric IgG4 and 250 kDa for heterotetrameric IgE antibody (Figure 4.14A).

Free light or heavy chain was not detected, suggesting that the antibody chains are assembled into whole antibody molecules. The molecular sizes correspond to the heavy and light chains suggesting that the secreted antibodies are properly folded and glycosylated.

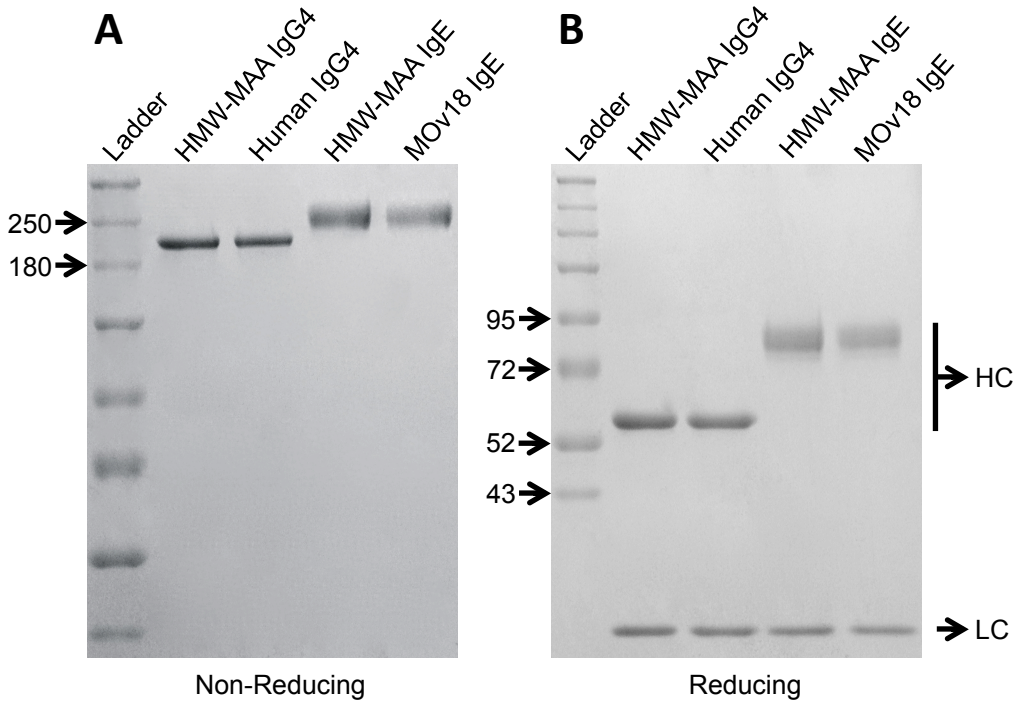


Figure 4.14: SDS-PAGE analysis of HMW-MAA specific IgG4 and IgE. Affinity purified HMW-MAA IgG4 and IgE antibodies were analysed under non-reducing (A) and reducing conditions (B), alongside with isotype controls human myeloma IgG4 and MOv18 IgE respectively, visualised by Coomassie staining. (HC=heavy chain, LC=light chain)

4.3.8 Flow cytometric assessment of affinity purified HMW-MAA IgE and IgG4

The functional characteristics of stably expressed and affinity column-purified recombinant HMW-MAA-specific IgE and IgG4, were tested by flow cytometry analysis of the both antigen and receptor binding activities of the antibodies. Both HMW-MAA-specific IgE and IgG4 antibodies bound to HMW-MAA+ A374 melanoma cells (Figure 4.15) but not HMW-MAA- primary melanocytes.

The receptor-binding activity of the antibodies were analysed by binding to the monocytic cell line U937, which expresses Fcγ receptors at low densities or RBL cells expressing both human and rat FcεRI (Dibbern et al, 2003). IgE bound to human FcεR1 receptor, expressed on RBL SX38 mast cells, and IgG4 bound to the surface of U937 cells, expressing Fcγ receptors (Figure 4.15).

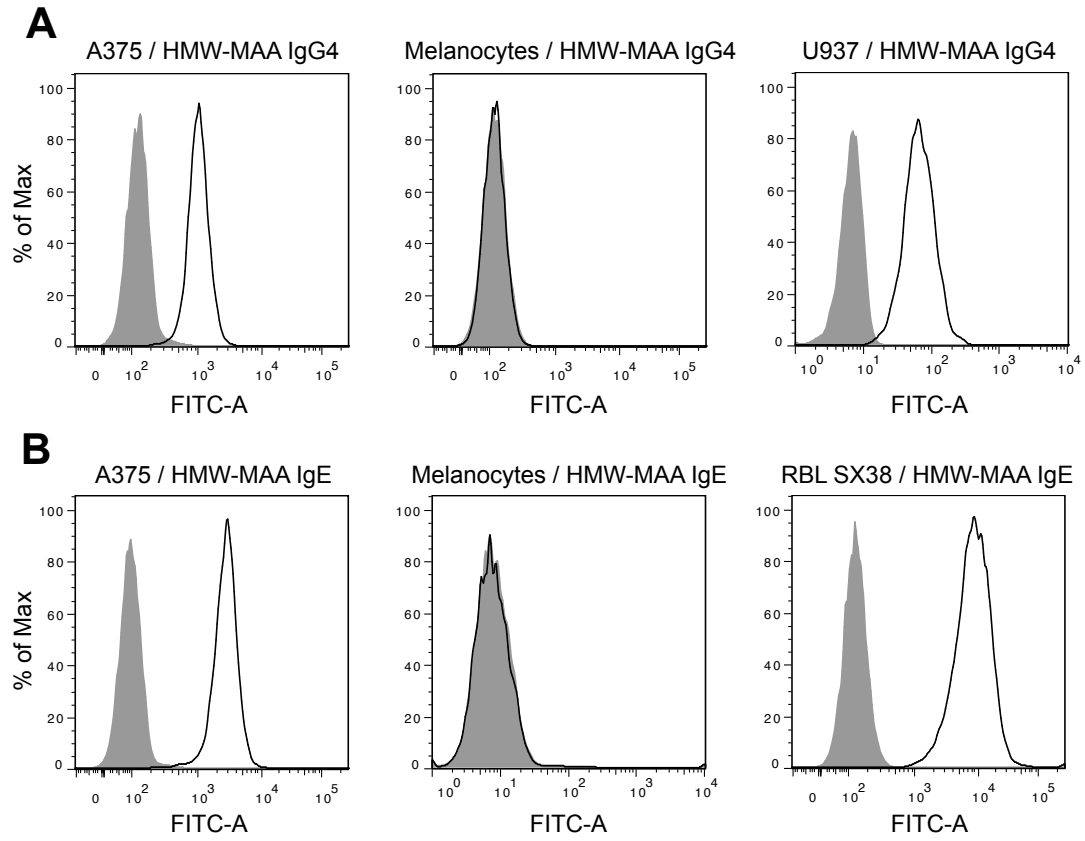


Figure 4.15: Flow cytometric assessment of HMW-MAA specific IgG4 and IgE. Recombinantly expressed HMW-MAA specific IgG4 (A) and IgE (B) antibodies show specific binding to native HMW-MAA present on the cell surface of A375 melanoma cells and no binding above background to primary melanocytes. The Fc fragments of IgG4 isotype demonstrate effector-binding to U937 monocytic cell line, expressing human Fcγ receptors. The IgE antibody isotype also represent comparable binding to RBL SX38 mast cells, expressing human FcεR1 receptor.

4.4 Discussion

Recombinant monoclonal antibodies (mAbs) have become a key tool for basic research and diagnosis of a number of human diseases. Over the last decade, antibody-based immunotherapies have been developed for the treatment of cancer, autoimmunity and inflammatory diseases (Leavy, 2010). The therapeutic antibodies infliximab and adalimumab, specific for tumour necrosis factor-alpha (TNFα), are sucessfully used to treat rheumatoid arthritis, as well as Crohn's disease and psoriasis. The human epidermal growth factor receptor 2 (HER2)- specific antibody trastuzumab and the

vascular endothelial growth factor A (VEGFA)-specific antibody bevacizumab, are used in the treatment of several types of cancer. The CD20-specific antibody rituximab is used to treat both rheumatoid arthritis and non-Hodgkin's lymphoma. This has resulted in a significant improvement in antibody production systems, allowing biopharmaceutical communities to reach grams per liter expression levels. However, in academia a costly and labour intensive process due to the lack of a suitable manufacturing platform, ensuring consistent antibody production, restricts the development of recombinant antibody material. Antibody expression systems representing co-transfection of vectors coding for either the heavy- or light-chain cDNA (Boel et al, 2000; Furtado et al, 2002; Koelsch et al, 2007; Madritsch et al, 2011; Tiller et al, 2008) or vectors hosting a dual antibody expression cassette (Braren et al, 2007; Hecker et al, 2011; Wiberg et al, 2006) have been exploited. However, these systems rely on restriction enzyme- and ligation-dependent cloning methods, which are time-consuming, prevent high-throughput analysis of antibody candidates and sometimes impractical due to the lack of compatible restriction sites. Therefore, I sought to generate an improved flexible system for the cloning and expression of antibodies of any species and isotype with any desired specificity.

First, to eliminate the requirement for co-transfection of two vectors, coding for the expression of heavy and light antibody chains independently, I constructed a dual expression cassette in a single mammalian expression vector thus halving the time and effort needed for DNA preparation and transfection procedure. An optimal ratio of heavy to light chain genes is important for efficient recombinant antibody production (Schlatter et al, 2005). Therefore I took advantage of the differing activities of the 2 enhancers in pVITRO1, placing the heavy chain cassette under the control of SV40 and the light chain cassette under the control of the more active CMV. Another advantage of vector pVITRO1 is the presence of hph gene, which confers resistance to Hygromycin

B in both *E. coli* and mammalian cells. This allows a selection step and generation of stable cell lines. To prevent the use of restriction enzyme- and ligation-dependent cloning methods, I took advantage of the Polymerase Incomplete Primer Extension (PIPE) cloning method, shown to be rapid, cost-effective, and highly efficient, and thus capable of supporting the high-throughput cloning of thousands of genes in parallel (Klock et al, 2008). Following basic primer design steps (Figure 4.5) for generation of overlapping sequences, I constructed the dual antibody expression cassette within pVITRO1 utilising the PIPE cloning method. To improve the system's flexibility to seamlessly exchange variable regions, I have designed universal primer pairs flanking the variable regions and successfully exchanged the specificity of an IgE antibody in a single day. This demonstrates that the system is suitable for high-throughput screening of antibody candidates derived from antibody discovery programs. Moreover, to provide constructs coding for the expression of different antibody isotypes with the same antigen specificity, and thus supporting the paralleled characterisation and comparative functional studies between different isotypes, I have shown a cloning procedure for exchanging the isotype of a HMW-MAA specific IgE to IgG4. These cloning experiments represent the system's simplicity to exchange variable- and/or constant-region antibody domains allowing cloning of different isotypes with any desired specificity.

Having successfully adapted and optimised the PIPE cloning method for our antibody needs, I aimed to demonstrate the capacity of the stable antibody expression system to produce fully functional antibodies, by expressing and characterising the HMW-MAA specific IgE and IgG4. A small-scale transfection in 293-F cells, followed by 2 weeks selection with Hygromycin B and generation of stable cell lines, enabled the expansion and large-scale production of antibodies into 1L shaker flask, 1L spinner or a 5L WAVE bioreactor. The anti-human IgE ELISA analysis of sample supernatants showed

10–25 mg/L HMW-MAA specific IgE expression levels within 30 days post-transfection, compared to 1 mg/L by the transient antibody expression system using the pSG vectors. Using a 5L WAVE bioreactor, 125 mg total amount of antibody was produced within 30 days. This quantity is sufficient to support *in vivo* animal model experiments. Similarly, the IgG4 ELISA results demonstrate that HMW-MAA-specific IgG4-producing stable cells reached 70 mg/L expression level within 2 weeks using 1L spinner. Although cloning the dual antibody cassette into pVITRO1 for expression in 293-F cells has facilitated high expression yields, the cassette can be transferred to any compatible expression vector consisting of two transcription units for use in alternative systems, if required.

Having optimised the stable expression system for large-scale production, I aimed to characterise the antibodies produced and demonstrate their functionality. The HMW-MAA specific IgE isotype was purified by affinity chromatography using IgG4-Fc-$(sFc\varepsilon RI\alpha)_2$ fusion protein and the IgG4 isotype was purified on a Protein-G column using an ÄKTA Prime system. The purity and size of the antibodies were assessed by SDS-PAGE analysis. Under non-reducing conditions, free light or heavy chain was not detected, indicating the antibody chains are assembled into whole antibody molecules. None of the purified antibodies showed significant contamination with other proteins, as judged by the absence of bands of molecular weight inconsistent with those expected for an IgE antibody polypeptide chains, indicating the affinity column purification was effective in yielding pure IgEs. The represented molecular sizes in SDS–PAGE suggested that the secreted antibodies are properly folded and glycosylated, in particular the extensively glycosylated IgE antibody. The biophysical properties and aggregative tendencies of the purified IgE and IgG4 isotypes were analysed by size-exclusion chromatography. The analysis showed no aggregation and confirmed the affinity column-purified product consists of monomeric antibodies.

Finally, the biological functions of the stably expressed and affinity column-purified recombinant HMW-MAA specific IgE and IgG4, were analysed by flow cytometry analysis to demonstrate both antigen and receptor binding activities. Flow cytometric analysis of A375 melanoma cells incubated with the HMW-MAA specific IgE and IgG4 antibody, showed comparable specific binding properties to native HMW-MAA and no binding above background to primary melanocytes. Furthermore, the IgE bound to human FcεR1 receptor, expressed on RBL SX38 mast cells, and IgG4 bound to the surface of U937 cells, expressing Fcγ receptors, demonstrating functional receptor-binding activity of the recombinant antibodies.

To summerise, I have developed a useful method for one-step assembly of antibody heavy- and light-chain cDNAs in a single mammalian expression vector. The DNA fragments assembled in this reaction do not rely on restriction enzymes and ligase-dependant methods, thus minimising the steps involved in the cloning procedure and allowing the reproducible generation of fully functional recombinant antibodies of any species and isotype with any desired specificity, at the level of tens of milligrams per litre. The combination of seamless one-day cloning of antibody genes in a single vector, combined with an easily scalable production process delivering sufficient material for large scale studies such as animal model experiments or pre-clinical studies provides an unbiased manufacturing platform compared to the currently available antibody expression methods in academia. This method enables the direct comparison of different antibody isotypes, that could readily be adapted for use in mechanistic studies of antigen-antibody interactions, and facilitates the parallel processing of a large panel of antibodies for identification of potential candidates for clinical applications.

5 IgE FOR THE TREATMENT OF SOLID TUMOURS

5.1 Introduction

Within the last decade, therapeutic monoclonal antibodies have been complementing the conventional treatments of some malignant diseases and have improved prognosis for many cancer patients. However, while more than half of these antibodies are approved for the treatment of blood malignancies, an antibody therapy is urgently needed for the treatment of solid, non-haematopoietic tumours.

IgG, the most abundant antibody class in the blood, is the only antibody class examined in the immunotherapy of cancer and nearly all antibodies developed in the clinic for cancer therapy to date are of the IgG1 subclass (Scott et al, 2012). This arises from the early work of Neuberger, showing that IgG1 was the most efficacious of nine different antibody classes in complement-mediated lymphoma cell killing by human PBMC *in vitro* (Bruggemann et al, 1987). Subsequent clinical trials with antibodies recognising the B cell marker CD20 supported the inference that IgG1 would be the subclass best suited for immunotherapy of patients with B cell malignancies such as non-Hodgkin's lymphoma (Alduaij & Illidge, 2011). However, poor tissue penetration of IgG antibodies and low affinity of IgGs for their receptors on immune cells may partly account for the weak immune responses observed and resulting poor performance of many IgG antibodies against solid tumours. IgE antibodies play a major role in the human allergic response, but are also key contributors to the body's defence against parasitic infections. IgE antibodies differ from the more abundant IgG isotypes, in that they are unable to fix complement and do not bind to the Fcγ receptors. They can be transported from the circulation into tissues, where, through their strong affinity for their receptors on immune cells, they are known to trigger powerful immune responses. The concentration of IgE in the serum of normal individuals is minute, and unlike IgG,

the presence of IgE in the blood is short-lived (half-life of 1.5 days) (Gould & Sutton, 2008; Gould et al, 2003; Ravetch & Kinet, 1991). Yet, IgE is sequestered in tissues and retained locally by powerful IgE receptor-expressing resident cells such as mast cells, macrophages and dendritic cells with a measured half-life of two weeks, proportionately longer than that of IgG (2-3 days) (Gould et al, 2003; Hellman, 2007). These properties of IgE antibodies may be redirected to enhance cytotoxicity and phagocytosis of tumour cells, as well as initiate IgE antibody-dependent antigen presentation by IgE receptor-bearing antigen-presenting cells such as dendritic cells, B cells and macrophages. Thus, passive and active immunity against solid tumours could act in conjunction in tissues such as the skin which is naturally populated with IgE effector cells. The strength of IgE-mediated immune responses in tissues, then, carries the expectation of increased potency as well as longevity of immune surveillance by IgE and effector cells against skin tumours. We have found that the results of *in vitro* experiments using IgG1 do not translate well to the solid tumour environment, where other mechanisms may prevail (Bracher et al, 2007; Karagiannis et al, 2008b; Karagiannis et al, 2007; Reali et al, 2001). We have demonstrated superior *in vivo* efficacy for the IgE subclass in comparison to IgG in the targeting of MOv18, an antibody directed against folate receptor, a tumour- specific antigen, in two murine xenograft models of human ovarian cancer (Gould et al, 1999; Karagiannis et al, 2003). Therefore, we believe the efficacy of cancer immunotherapy may be improved using IgE isotype in place of the currently conventional IgG and aimed to engineer an IgG1 and IgE against a melanoma-specific antigen, the High Molecular Weight Melanoma-Associated Antigen (HMW-MAA) (Chan & Murphy, 1999; Hafner et al, 2005; Kang et al, 2000; Luo et al, 2006), for comparative functional studies between the two isotypes. The work represented in this chapter is a result of a fruitful collaboration with Dr. Panos Karagiannis.

5.2 Methods

5.2.1 Cloning of IgG1 expression construct

The pVitroMAA-IgE vector was PCR linearised as previously described (see Section 4.5), and subsequently treated by *Dpn I* (NEB) according to the manufacturer's instructions to destroy the vector template. The human Cγ1 region was amplified, using previously optimised reaction conditions (see Section 4.4) and 10 seconds extension time, from an existing pSG vector (Karagiannis et al, 2009) using primer pair Gam1_Fwd and Gam1_Rev (Table 2.2), for generation of vector fragment terminal end-homology. The *Dpn I* treated linearised vector and unpurified Cγ1 PCR product were mixed in 1:1 (v/v) ratio, followed by 30 minutes incubation at 60°C and 2 µl from the mixture transformed into XL1-Blue competent cells. The newly generated vector was named pVitroMAA-IgG1, coding for the expression of chimeric HMW-MAA specific IgG1 antibody.

5.2.2 Immunofluoresence staining of A375 cells

A375 metastatic melanoma tumour cells were seeded in LabTek glass chamber slides (Nunc) at a density of 2 x 10^4 cells/well. Cells were washed in PBS and fixed in 4% formaldehyde PBS for 15 min and then incubated with PBS-T, 10% goat serum, 0.05% Tween for 25 min at room temperature. HMW-MAA specific IgG1 and IgE or control NIP (hapten specific) IgG1 and IgE antibodies were incubated for 45 min with A375 cells at a concentration of 0.01µg/µl. Cell-bound antibodies were detected with a secondary goat anti-human IgG or goat anti-human IgE antibody conjugated to FITC (Jackson ImmunoResearch; 1:250). Nuclei were stained with Hoechst dye for 3 mins. All washing steps were done in PBS and the final wash was performed with dH_20. Cells were then mounted in Mowiol (Sigma) mounting medium. Fluorescence microscopy was performed on a Zeiss AxiovertZ.1 (40x objective) upright microscope. Acquisition

and analysis was performed with an AxioCamMR3 and AxioVision Software (Carl Zeiss).

5.2.3 Degranulation assay

RBL SX-38 cells were seeded at a concentration of $2x10^4$ cells/well in 100µl media onto 96-well round bottom plates (96 Nucleon, Nunc, Roskilde, Denmark) and incubated in a 5% CO_2 humidified incubator at 37°C. 24 h later, the cells were passively sensitised with 100ng/ml of HMW-MAA IgE or control anti hapten specific IgE (NIP) antibody and incubated for 2 hours. Cells were then washed twice with stimulation buffer (Hank's Buffered Salt Solution, HBSS supplemented with 1% BSA) and left untreated (control) or triggered with either $2x10^3$ A375 tumour cells per well, 100 ng/ml of polyclonal rabbit anti human-IgE (Dako, Ely, UK) antibody, or stimulation buffer alone for 30 mins at 37°C in a humidified incubator. Control supernatants obtained from untreated wells were considered background degranulation and the HBSS containing 0.1% Triton-X100 as a total release (100% degranulation). Degranulation was terminated by placing the experiment on ice and 50µl of supernatant was transferred to 96 polystyrene black MicroWell™ Plates (Nunc, Roskilde, Denmark) for analysis. Quantification was determined by measuring β- hexosaminidase release using a fluorogenic substrate (4-methylumbelliferyl-N-acetyl-β-D-glucosaminide) prepared according to a standard protocol (Casal et al, 2003; Linko-Lopponen & Makinen, 1985). Briefly, supernatants were incubated with an equal amount of fluorogenic substrate at 37°C in the dark for 2 hour and then quenched using 0.5M Tris. Finally fluorescence was read on an Omega microplate reader (BMG Labtech, Aylesbury, UK) using excitation and emission filters at 360nm and 405nm. % of degranulation was calculated as relative to 100% degranulation with Triton-X100.

5.2.4 Three-color flow cytometric assay

CFSE, a green fluorescence derived intracellular dye, was used to stain A375 tumour cells prior to incubation with U937 monocytic cells. After the incubation, U937 cells were stained with anti-CD89-PE and 7-AAD indicating effector cells and dead cells, respectively. A375 cells were stained 24 h prior to assays with 5.0 μM CFSE (5-(and 6-) carboxyfluorescein diacetate succinimidyl ester, Molecular Probes, Eugene, OR) in PBS for 10 min at 37^{o}C, washed in DMEM medium supplemented with 10% FCS, 2 mM L-glutamine, and returned to normal culture conditions. The following day, CFSE-labelled tumour cells were washed, mixed with unstained U937 effector cells at E:T cell ratio of 4:1 with or without antibodies, followed by incubation for 3 h at 37^{o}C. Antibodies were tested at a concentration of 4 μg/mL. CFSE-labelled tumour cells were detected in FITC (530/30 nm band pass filter and a 502 long pass filter), CD89-PE-labelled monocytic effector cells in PE (585/42 nm band pass filter and a 556LP) and 7-AAD^{+} dead cells in APC (660/20 nm band pass filter) channels, while control samples were set for compensation adjustments between CFSE and PE. Two dual colour flow cytometric dot plots were generated to calculate ADCC and ADCP as previously described (Bracher et al, 2007; Karagiannis et al, 2008a; Karagiannis et al, 2008b; Karagiannis et al, 2007). Briefly, one dot plot depicted CFSE^{+} tumour cells on the x-axis and 7-AAD^{+} cells on the y-axis, allowed quantification of double positive cells (CFSE^{+}/7-AAD^{+} cells) indicating tumour targets killed externally by effector cells (ADCC, cytotoxicity). The second dot plot depicted CFSE^{+} tumour cells on the x-axis and CD89-PE^{+} effector cells on the y-axis in order to quantify total CFSE^{+} tumour cells and the number of tumour cells material present within PE^{+} effector cells, indicating phagocytosis (ADCP) by effector cells (CFSE^{+}/PE^{+} cells). No antibody or 4μg of Mov18 antibody isotypes directed against FRα, which is not expressed on A375 tumour cells, were used as controls and assay conditions ran in triplicates.

5.3 Results

5.3.1 Cloning, expression and purification of HMW-MAA specific IgG1

In chapter 4, I have described the construction of a dual antibody expression cassette in pVITRO1 and the subsequent PIPE cloning of HMW-MAA specific IgE and IgG4 expression vectors. A stable cell line expressing the HMW-MAA specific IgE has been generated and frozen in liquid nitrogen. Thus to support the parallel characterisation and comparative functional studies between IgE and IgG1 isotypes, I proceeded with PIPE cloning of HMW-MAA specific IgG1 expression vector (Figure 5.1).

Having successfully cloned the pVitroMAA-IgG1 vector, I continued with the expression of the HMW-MAA specific IgG1 antibody. 293-F cells were transfected as previously described (Methods 4.2). The cells were kept under Hygromycin selection for 2 weeks and a stable cell line was generated. Simultaneously, frozen cells expressing the HMW-MAA specific IgE were thawed and cultured to recover. The IgG1 and IgE expressing cell lines were expanded into 1L shaker flasks and sample supernatants collected after 12 days for antibody quantification by anti-human IgE/IgG1 ELISA as described previously (Gilbert et al, 2011; McCloskey et al, 2007). The ELISA analysis showed 13mg/L IgG1 and 10mg/L IgE expression levels (Figure 5.2), consistent with previous measurements using shaker flasks. This result further confirmed that the stable antibody expression system was capable of generating material in quantities that could support animal model experiments, enabling me to continue with the purification process.

The HMW-MAA specific IgE isotype was purified by affinity chromatography using IgG4-Fc-$(sFc\varepsilon RI\alpha)_2$ fusion protein, as previously described (Methods 3.2). The HMW-MAA specific IgG4 isotype was purified on a Protein-G column as previously described

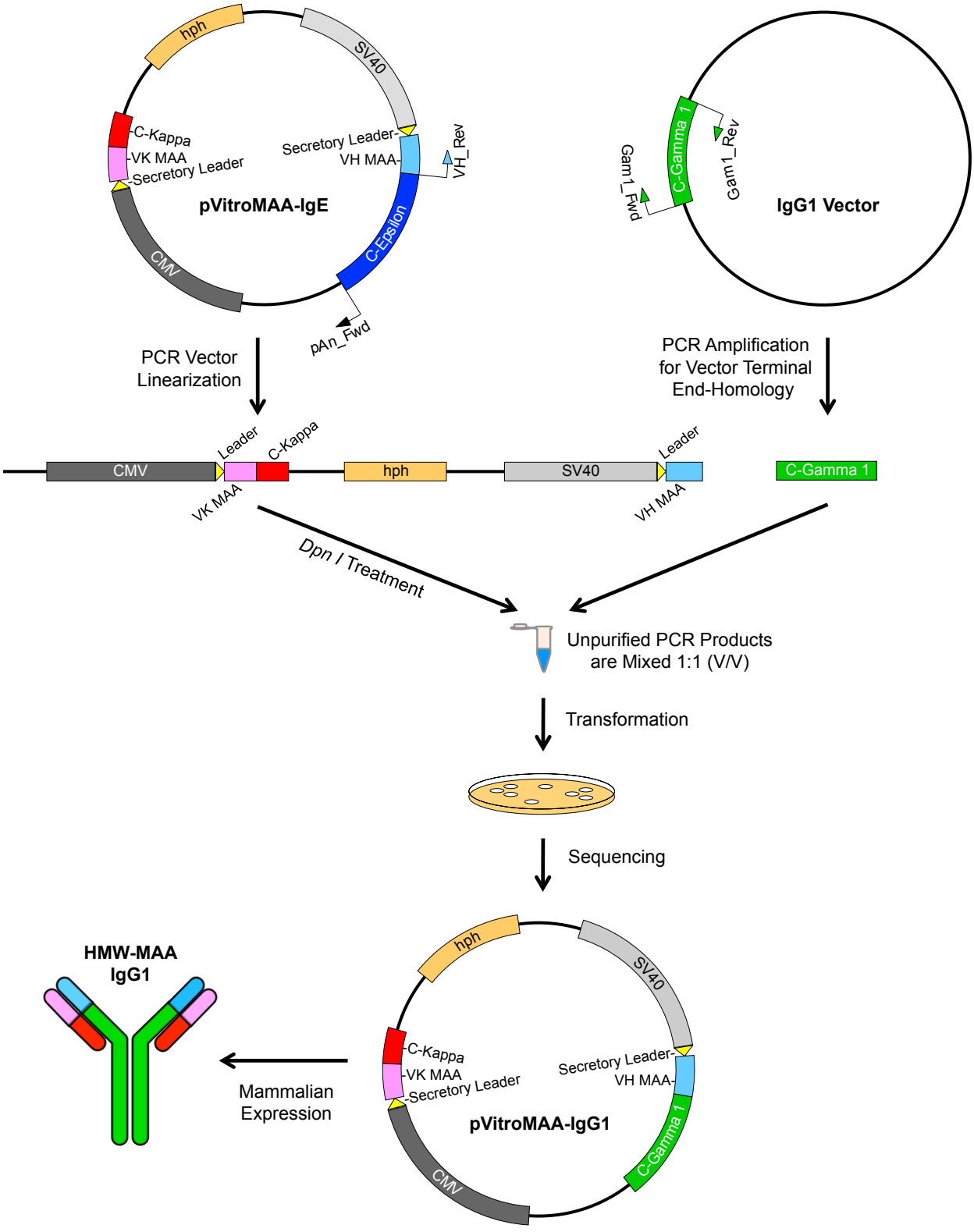


Figure 5.1: Schematic representation of HMW-MAA specific IgG1 cloning. pVitroMAA-IgE expression vector is PCR linearised by C-Epsilon region flanking primers and subsequently *Dpn I* treated. Simultaneously, C-Gamma 1 region is PCR amplified for generation of vector terminal end-homology. The unpurified *Dpn I* treated linearised vector is mixed 1:1 (v/v) with unpurified C-Gamma 1 PCR product. The single-stranded DNA fragments anneal directionally across the complementary sequences and nicks and gaps are repaired *in vivo* after transformation, generating pVitroMAA-IgG1 expression vector.

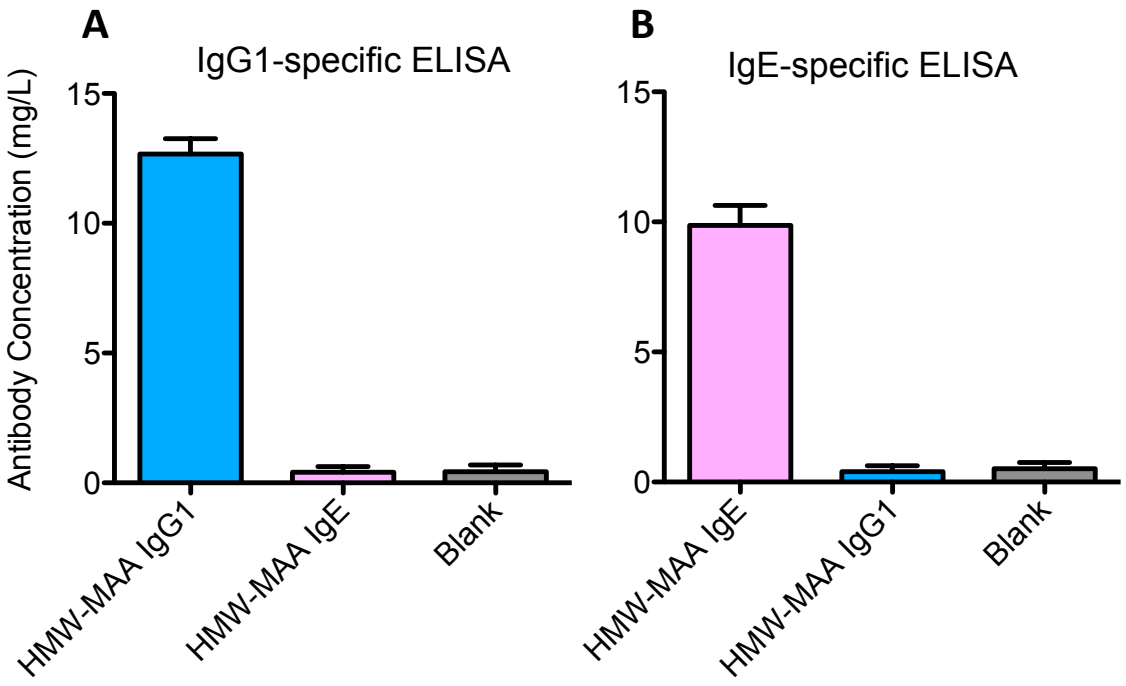


Figure 5.2: Quantification of HMW-MAA specific IgG1 and IgE expression levels by ELISA. Supernatants from recombinantly expressed antibodies were analysed by IgG1 (A) and IgE (B) isotype-specific ELISA after 12 days incubation of stable cell lines in shaker flasks. Samples from IgE expressing cells, used as a negative control, did not give a signal above background levels on the IgG1 specific ELISA. Similarly supernatants from IgG1 expressing cells gave no signal on the IgE specific ELISA. The concentration of antibody in mg/L was determined by reference to a standard curve and results represent the mean of triplicate readings ± SD.

5.3.2 Characterisation of HMW-MAA specific IgG1 and IgE

The biophysical properties of affinity column-purified HMW-MAA specific IgE and IgG1 and previously characterised isotype controls MOv18 IgE and IgG1 (Karagiannis et al, 2003) were analysed by size-exclusion chromatography, as previously described (Methods 4.2). The size-exclusion chromatography analysis showed no aggregation and confirmed the affinity column-purified product consists of monomeric antibodies (Figure 5.3).

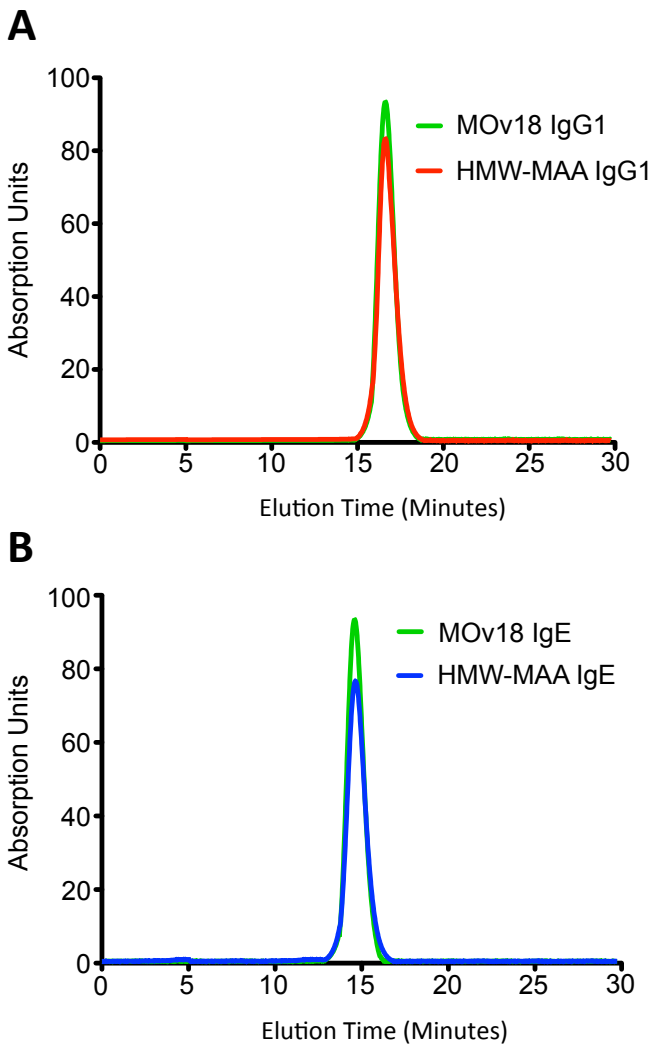


Figure 5.3: Size exclusion chromatography analysis of HMW-MAA specific IgG1 and IgE. Elution profiles of recombinantly expressed and affinity purified HMW-MAA specific IgG1 (A) and IgE (B) antibodies, compared to previously-characterised isotype controls MOv18 IgG1/IgE respectively, from Superdex™ 200 gel filtration. The antibody profiles correspond to isotype controls and confirm the affinity purified products consist of monomeric antibodies.

The size and purity of affinity column-purified antibodies HMW-MAA specific IgE and IgG1 and isotype controls MOv18 IgE and IgG1 respectively, were assessed by SDS-PAGE analysis as previously described (Methods 4.2). Under reducing conditions (Figure 5.4), protein bands corresponding to molecular size between 52-72 kDa of the protein ladder represented the IgG1 heavy chains. Lower bands corresponding to the antibody light chains were also visualised. Similarly, protein bands of molecular size between 72-95 kDa and lower bands were visualised suggesting the heavy and light chains respectively, of the IgE antibodies. Under non-reducing conditions, molecular

sizes of the visualised protein bands were found to be in the range of 180-250 kDa for the heterotetrameric IgG1 and 250 kDa for heterotetrameric IgE antibody. Free light or heavy chain was not detected, suggesting that the antibody chains are assembled into whole antibody molecules. Thus, the represented molecular sizes in SDS–PAGE suggest that the secreted antibodies are properly folded and glycosylated, in particular the extensively glycosylated IgE antibody.

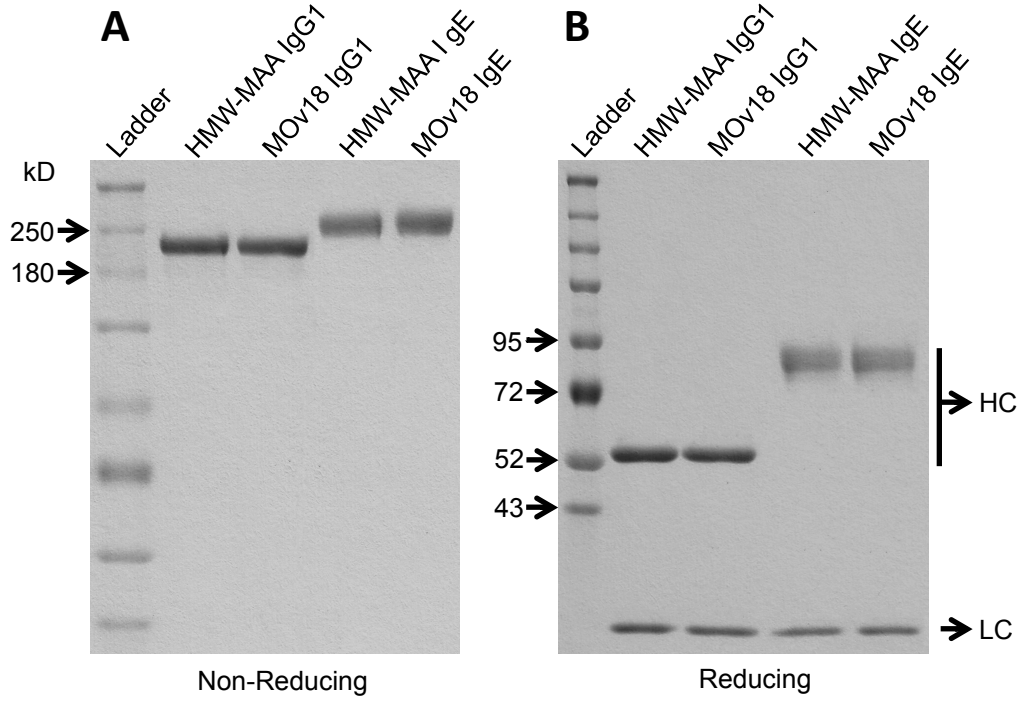


Figure 5.4: SDS-PAGE analysis of HMW-MAA specific IgG1 and IgE. Affinity purified HMW-MAA specific IgG1 and IgE antibodies were analysed under non-reducing (A) and reducing conditions (B), alongside with previously-characterised isotype controls MOv18 IgG1/IgE respectively, visualised by Coomassie staining. The represented antibody molecular masses are identical to isotype controls and show the secreted antibodies are properly folded and glycosylated.

The functional studies of affinity column-purified recombinant HMW-MAA specific IgE and IgG1, were pursued by flow cytometry analysis to demonstrate both antigen and receptor binding activities of the antibodies. The flow cytometric assessments were used to compare antigen specificity of HMW-MAA IgE and IgG1 to native HMW-

MAA as presented on the cell surface of A375 melanoma cells and to primary human melanocytes, normally present in the skin of healthy volunteers. The receptor-binding activity of the IgG1 antibody isotype was analysed by binding to the surface of monocytic cell line U937, which expresses Fcγ receptors at low densities. The receptor-binding activity of the IgE antibody isotype was analysed by binding to RBL SX38 mast cells expressing both human and rat FcεRI (Dibbern et al, 2003). The flow cytometric experiment was performed as previously described (Methods 4.2). Flow cytometric analysis (Figure 5.5) of A375 melanoma cells incubated with the HMW-MAA specific IgE and IgG1 antibody, shows a clear right shift of fluorescence intensity, suggesting that both antibodies had comparable specific binding properties to native HMW-MAA as detected by >95% positive populations (gating above isotype controls MOv18 IgE and IgG1) and no binding above background to primary melanocytes. Furthermore, the IgE bound to human FcεR1 receptor, expressed on RBL SX38 mast cells, and IgG1 bound to the surface of U937 cells, expressing Fcγ receptors, demonstrated functional receptor-binding activity of the recombinant antibodies.

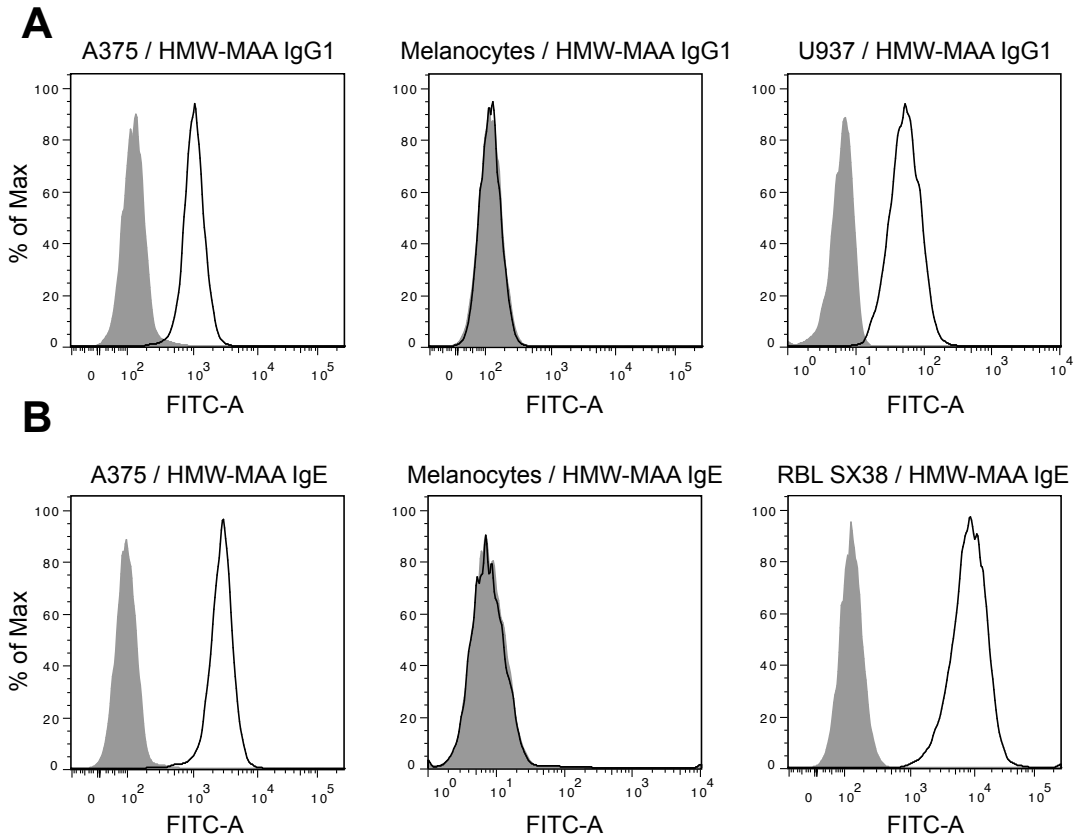


Figure 5.5: Flow cytometric assessment of HMW-MAA specific IgG1 and IgE. Recombinantly expressed IgG1 (A) and IgE (B) antibodies show specific binding to native HMW-MAA present on the cell surface of A375 melanoma cells and no binding above background to primary melanocytes. The Fc fragments of IgG1 isotype demonstrate effector-binding to U937 monocytic cell line, expressing human Fcγ receptors. The IgE antibody isotype also represent comparable binding to RBL SX38 mast cells, expressing human FcεR1 receptor. Antibody binding was detected using a goat anti-human IgG-FITC (A) or IgE-FITC (B) antibody.

5.3.3 Immunofluoresence staining of A375 cells grown on glass chamber slides

Having successfully analysed antigen and receptor binding activities of HMW-MAA specific IgG1 and IgE antibodies by flow cytometry, I aimed to confirm their specific binding to HMW-MAA as presented on the cell surface of A375 cells by immunofluorescence microscopy.

Binding of the HMW-MAA specific IgG1 and IgE antibodies to the surface of the A375 tumour cells was confirmed by immunofluorescence microscopy, while a hapten specific isotype control, NIP-IgE and NIP-IgG1 did not show binding above background (Figure 5.6). Therefore, the flow cytometry and immunofluorescence of

HMW-MAA specific IgG1 and IgE confirmed the antibodies recognised the expected tumour target and immune effector cells.

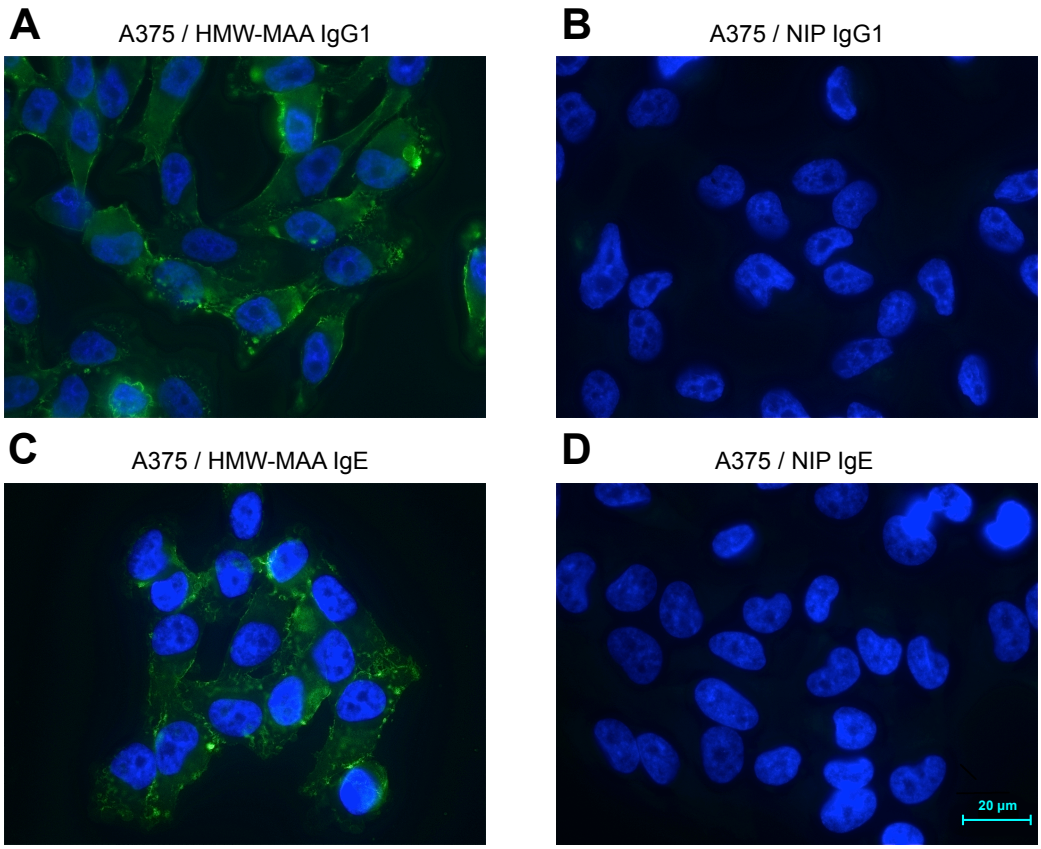


Figure 5.6: Immunofluorescence staining of HMW-MAA specific IgG1 and IgE to A375 cells. Antibodies bound to A375 melanoma tumour cells were detected by goat anti-human IgG/IgE-FITC antibodies. The immunofluorescence confirms specific binding of HMW-MAA IgG1 (A) and HMW-MAA IgE (C) to A375 cells and no background binding with isotype controls hapten specific NIP-IgG1 (B) or NIP-IgE (D). Images were captured using a 63x oil objective. Scale bar = 20 µm.

5.3.4 HMW-MAA specific IgE effector cell activation

Flow cytometry and immunofluorescence experiments, showed the binding of both IgG1 and IgE to the expected tumour target and immune effector cells. Next, I sought to examine the ability of HMW-MAA specific IgE antibody to trigger functional degranulation due to the recognition of HMW-MAA expressed on tumour cells and cross-linking of the human high affinity IgE (FcεRI) receptors, expressed on RBL SX-38 mast cells (Dibbern et al, 2003), in an antigen-dependent manner. This was performed using mast cell degranulation assay, measured by β-hexosaminidase release depicting mast cell activation.

The degranulation assay showed that the HMW-MAA specific IgE antibody alone did not induce degranulation (Figure 5.7). However, the antibody potentiated β-hexosaminidase release of RBL SX-38 cells following stimulation with a polyclonal anti-human IgE antibody. The HMW-MAA specific IgE induced significant degranulation of RBL-SX38 cells following stimulation with $2x10^3$ A375 cells (used for titration, data not shown), expressing HMW-MAA. This data suggests that IgE exhibits an intrinsic potential to cross-link the FcεRI and activate effector cells in an antigen-dependent manner. Furthermore, this represents the use of recombinant human IgE antibodies of defined specificity as a useful tool to investigate mechanisms underlying IgE-mediated reactions.

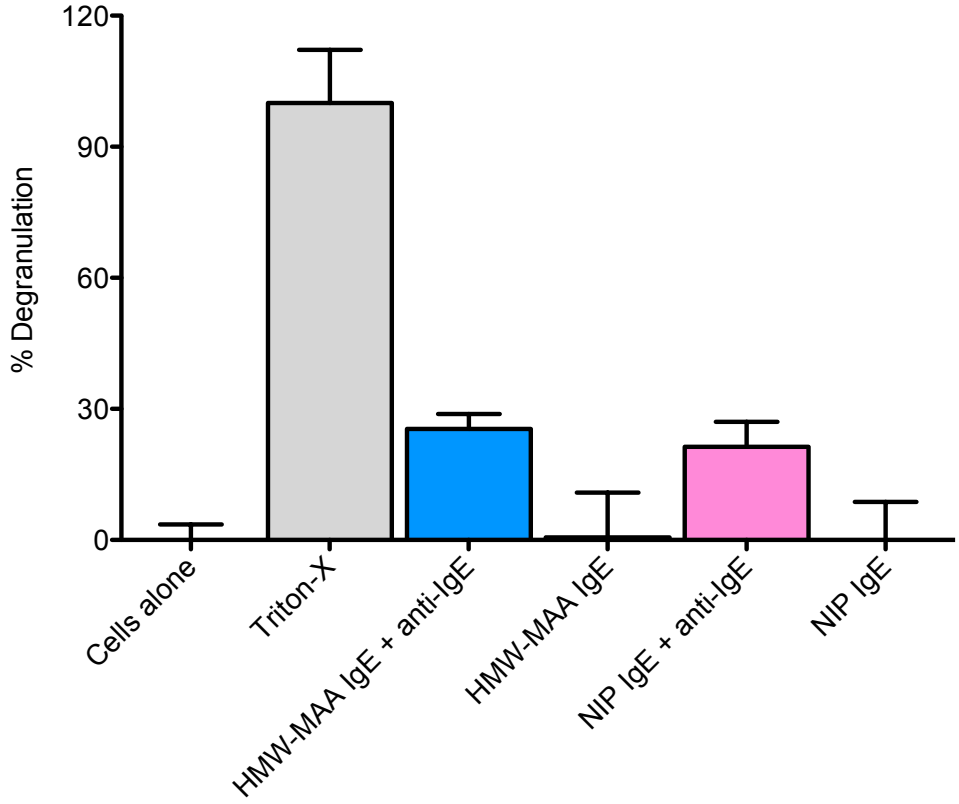


Figure 5.7: Degranulation of mast cells by HMW-MAA specific IgE. Degranulation (%) was measured by β-hexosaminidase release depicting mast cell activation, relative to Triton X-100. Minimal degranulation was seen with cells alone, control antibody NIP-IgE or HMW-MAA specific IgE alone. Significant degranulation is observed with samples given HMW-MAA specific IgE + polyclonal anti-IgE and NIP-IgE + polyclonal anti-IgE. Scale bars indicate mean values ± SD of n = 3 experiments, all conditions were tested in triplicate. Figure courtesy of Debra Josephs (PhD student, KCL).

5.3.5 Flow cytometric ADCC/ ADCP assay of HMW-MAA specific IgE and IgG1

Having observed that the HMW-MAA specific IgE is effective in cross-linking the FcεRI and activating effector cells in an antigen-dependent manner, we aimed to examine the ability of IgE and IgG1 to directly signal tumour cell death. We adapted a modified three-color flow cytometric assay (Bracher et al, 2007; Karagiannis et al, 2008a; Karagiannis et al, 2008b; Karagiannis et al, 2007) to simultaneously measure tumour cell cytotoxicity (ADCC) and phagocytosis (ADCP) of A375 melanoma cells, where U937 monocytic cells were employed as effector cells.

The three-color flow cytometric assay showed that after 3 hours in culture, U937 cells mixed with HMW-MAA specific IgG1 and A375 mediated significant levels of ADCP above that seen with samples incubated with the isotype control MOv18 IgG1 (23.30 *vs.*

8.34%; Figure 5.8A). However, U937 cells mixed with HMW-MAA specific IgE and A375 mediated predominantly ADCC above that seen with samples incubated with the isotype control MOv18 IgE (18.23 vs. 5.74%; Figure 5.8B). In addition, incubation with HMW-MAA specific IgE induced only minimal tumour cell ADCP compared to control MOv18 IgE (6.22 vs. 8.79%; Figure 5.8B). Thus, the flow cytometric ADCC/ADCP assay measurements confirmed that HMW-MAA specific IgE mediated significant levels of ADCP of A375 tumour cells by monocytic cells ($p<0.001$, Two-way Anova, with modified Bonferroni post-test).

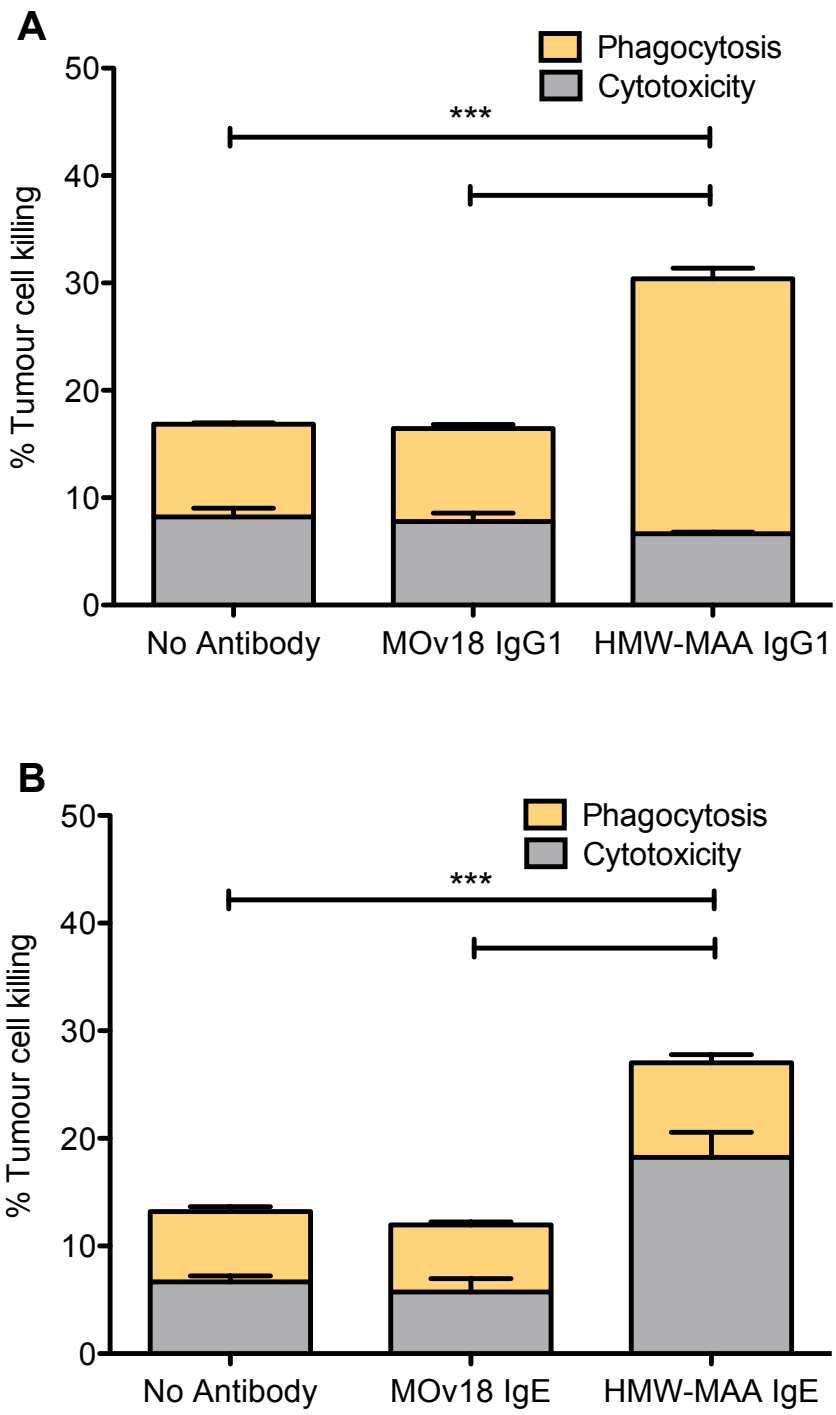


Figure 5.8: Three-colour flow cytometric tumour cell killing assessment of HMW-MAA specific IgG1 and IgE. Quantification of HMW-MAA IgG1-mediated (A) or IgE-mediated (B) A375 tumour cell killing by U937 monocytes is measured by ADCC/ ADCP assay. HMW-MAA specific IgG1 induces tumor cell killing via phagocytosis and HMW-MAA IgE kills tumour cells via cytotoxicity. Cytotoxicity: grey bars; phagocytosis: gold bars. Results are means ± SD of three independent experiments. (n=5; *p<0.05; **p<0.01; ***p<0.001; ns: p>0.05). Figure courtesy of Panos Karagiannis (PhD student, KCL).

5.3.6 Human melanoma xenograft mouse model

Ultimately, the efficacy of all potential therapeutics is tested *in vivo* using relevant models. The murine xenograft model of human ovarian cancer, shown to be very effective in comparing IgG1 and IgE (Gould et al, 1999; Karagiannis et al, 2003), represented a suitable model for targeting solid tumours. To study the potential tumour growth restricting capacity of the HMW-MAA specific IgE and IgG1 antibodies, we used this established immunodeficient mouse (NOD/SCID $\gamma^{-/-}$ mouse of BALB/c background) model of human melanoma.

5 x 10^5 A375 melanoma cells per mouse were injected subcutaneously in the flank. Following tumour challenge and engraftment of human PBLs to provide human effector cells, antibodies were administered weekly at 10 mg/kg doses by the intravenous route. The treatment with weekly doses of HMW-MAA specific IgE resulted in severely-restricted melanoma tumour growth over a period of 30 days, while tumours in mice treated with the corresponding HMW-MAA specific IgG1 antibody were smaller than the control antibodies MOv18 IgE and IgG1 against the tumour antigen FRα, which is not expressed on A375 melanoma cells (Figure 5.9). Xenografts given no antibody treatments grew exponentially up to 750mm^3 over the same time period. These initial experiments suggest that the HMW-MAA specific IgE antibody and to a lesser extent, HMW-MAA specific IgG1, are capable of mounting anti-tumoural responses *in vivo*.

Further immunohistochemical evaluations of the melanoma tumour marker HMW-MAA, normally expressed by A375 melanoma cells in culture, were performed on previously excised and snap-frozen tumours. Expression of the HMW-MAA on A375 subcutaneous melanoma tumours grown in NOD/SCID $\gamma^{-/-}$ mice was confirmed (Figure 5.10), suggesting the A375 melanoma xenograft model may be used to evaluate the therapeutic potential of monoclonal antibodies recognising this antigen. This is very

advantageous for the future evaluation of antibody candidates derived from our antibody discovery program (described in details in chapter 6).

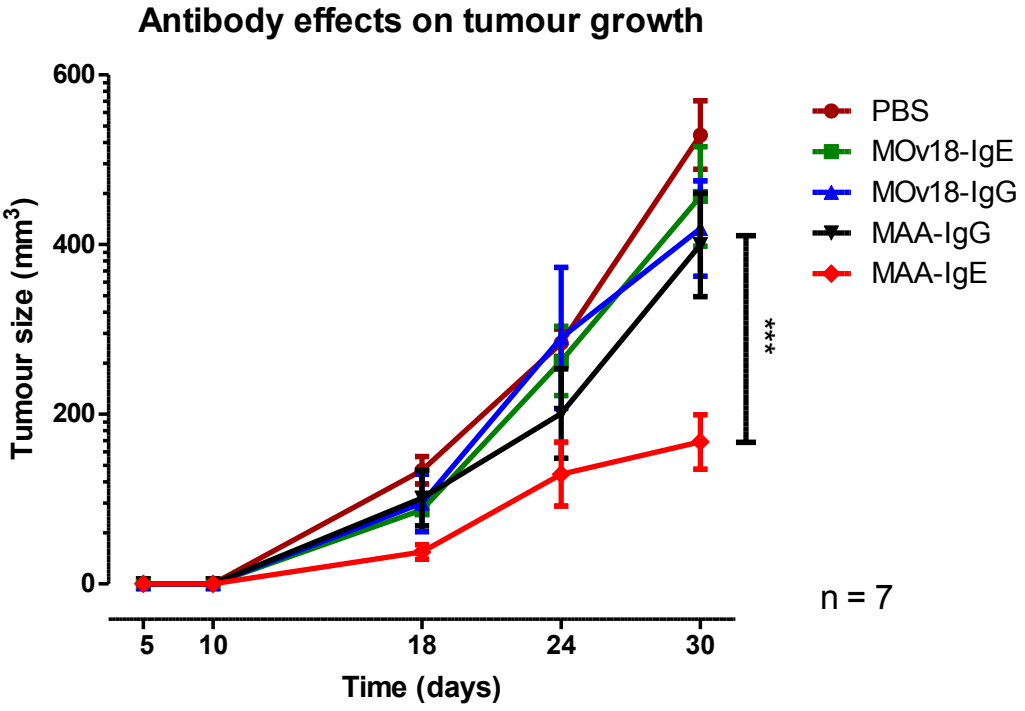


Figure 5.9: Tumour growth restriction efficacy of IgG1/IgE in a subcutaneous melanoma tumour model. Monitoring subcutaneous growth (mm^3) of melanoma tumours in NOD/SCID $\gamma^{-/-}$ mice at different time points following tumour challenge and engraftment of human PBLs as effector cells. Weekly treatment of HMW-MAA specific IgE severely restricted melanoma tumour growth, compared to mice treated with HMW-MAA specific IgG1 and control antibodies MOv18-IgG1/IgE. Figure courtesy of Panos Karagiannis (PhD student, KCL).

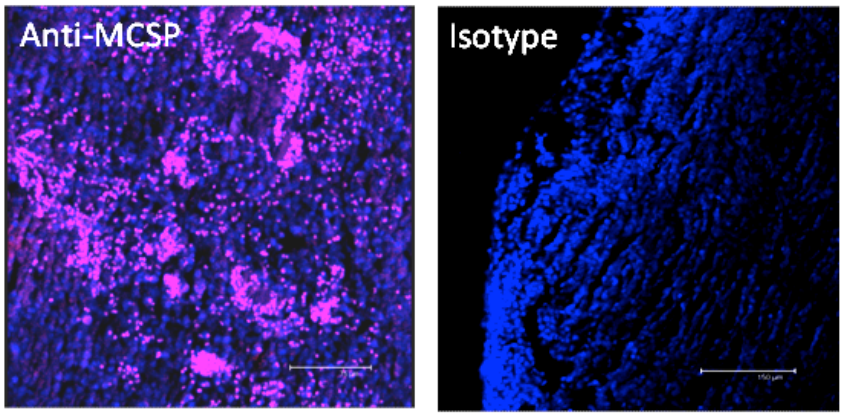


Figure 5.10: Immunohistochemical analysis of A375 metastatic melanoma cells. Subcutaneous tumour model of A375 metastatic melanoma cells, labelled for expression of melanoma marker HMW-MAA (left) and mouse IgG isotype control antibody (right). Images were captured using a 10x objective. Scale bar = 150 μm. Figure courtesy of Panos Karagiannis (PhD student, KCL).

5.4 Discussion

Some tumour antigen-specific monoclonal IgG antibodies have already been approved for therapeutic use and nearly all antibody therapies developed to date have relied on only one of the nine human antibody classes, namely IgG1 (Scott et al, 2012). This arises from the early work of Neuberger, showing that IgG1 was the most efficacious of nine different antibody classes in complement-mediated lymphoma cell killing by human PBMC *in vitro* (Bruggemann et al, 1987). However, it is known that IgG diffuses inefficiently into solid tissues, and binds with low affinity to receptors on immune effector cells.

IgE antibodies differ from the more abundant IgG isotypes, in that they can be transported from the circulation into tissues, where, through their strong affinity for their receptors on immune cells, they are known to trigger powerful immune responses. We have found that the results of *in vitro* experiments using IgG1 do not translate well to the solid tumour environment, where other mechanisms may prevail (Bracher et al,

2007; Karagiannis et al, 2008b; Karagiannis et al, 2007; Reali et al, 2001). We have demonstrated superior *in vivo* efficacy for the IgE subclass in comparison to IgG in the targeting of MOv18, an antibody directed against folate receptor, a tumour- specific antigen, in two murine xenograft models of human ovarian cancer (Gould et al, 1999; Karagiannis et al, 2003). Therefore, we aimed to engineer an IgG1 and IgE antibody against a melanoma-specific antigen, the High Molecular Weight Melanoma-Associated Antigen (HMW-MAA) (Chan & Murphy, 1999; Hafner et al, 2005; Kang et al, 2000; Luo et al, 2006), for comparative functional studies between the two isotypes.

Having successfully adapted and optimised the PIPE cloning method for swapping constant regions in chapter 4, I seamlessly exchanged human Cε in vector pVitroMAA-IgE with human Cγ1 and created vector pVitroMAA-IgG1, coding for the expression of HMW-MAA specific IgG1. Following a small-scale transfection in 293-F cells and 2 weeks selection with Hygromycin B, a stable cell line expressing HMW-MAA specific IgG1 was generated. Cell expressing IgG1 and IgE were expanded into 1L shaker flasks and after 12 days incubation, 13mg/L IgG1 and 10mg/L IgE expression levels were observed. The antibodies were purified by affinity chromatography and assessed by SDS-PAGE analysis. Under non-reducing conditions, free light or heavy chain was not detected, indicating the antibody chains are assembled into whole antibody molecules. None of the purified antibodies showed significant contamination with other proteins, as judged by the absence of bands of molecular weight inconsistent with those expected for an IgE or IgG1 antibody polypeptide chains, indicating the affinity purification was effective in yielding pure antibodies. The represented molecular sizes in SDS–PAGE suggested that the secreted antibodies are properly folded and glycosylated, in particular the extensively glycosylated IgE antibody. The biophysical properties and aggregative tendencies of the purified IgE and IgG1 isotypes were analysed by size-exclusion chromatography. The analysis showed no aggregation and confirmed the affinity

column-purified product consists of monomeric antibodies. This is an example of the effectiveness of our antibody cloning system in swapping C regions, simplifying the subsequent antibody production in sufficient quantities, capable of supporting the parallel characterisation and comparative functional studies between different isotypes.

The antigen and receptor binding activities of IgE and IgG1 were analysed by flow cytometry. Flow cytometric analysis of A375 melanoma cells incubated with the HMW-MAA specific IgE and IgG1 antibody, showed comparable specific binding properties to native HMW-MAA and no binding above background to primary melanocytes. Furthermore, the IgE bound to human FcεR1 receptor, expressed on RBL SX38 mast cells, and IgG1 bound to the surface of U937 cells, expressing Fcγ receptors, demonstrating functional receptor-binding activity and recognition of immune effector cells. The binding of the HMW-MAA specific IgG1 and IgE antibodies to the surface of the A375 tumour cells was also confirmed by immunofluorescence microscopy.

Following flow cytometry and immunofluorescence experiments, we examined the capacity of the HMW-MAA specific IgG1 and IgE antibodies to target and kill tumour cells, by employing Fc receptor-mediated effector cell activation in *in vitro* assays such as functional degranulation and ADCC/ADCP assays. In a functional degranulation assay measuring % β-hexosaminidase release by RBL SX-38 mast cells expressing human FcεRI, the HMW-MAA specific IgE antibody alone did not potentiate β-hexosaminidase release. However, the antibody induced significant degranulation of RBL SX-38 cells following stimulation with a polyclonal anti-human IgE antibody, demonstrating the ability of this antibody to activate immune effector cells through engagement of its high affinity receptor. Furthermore, both antibodies were capable of activating immune effector cells to kill cancer cells *in vitro* with similar effectiveness,

but each by different mechanisms: the chimaeric IgG1 activated human monocytes to kill tumour cells by ADCP, while the IgE mediated ADCC of tumour cells.

Finally, an immunodeficient mouse model of human melanoma grown subcutaneously in NOD/SCID $\gamma^{-/-}$ mice of BALB/c background was used to assess the ability of the melanoma antigen-specific antibodies to restrict tumour growth *in vivo*. The murine xenograft model of human ovarian cancer has been shown to be very effective in comparing IgG1 and IgE in targeting solid tumours (Gould et al, 1999; Karagiannis et al, 2003) and represented a suitable model of human melanoma. Using this subcutaneous *in vivo* model of melanoma and engraftment of human immune effector cells in these mice, treatment with weekly doses of HMW-MAA specific IgE (10mg/kg) resulted in severely-restricted melanoma tumour growth over a period of 30 days compared to those treated with the corresponding IgG1 at the same doses and to those given non-specific antibody controls. Furthermore, immunohistochemical evaluations confirmed the melanoma tumour lesions were positive for the HMW-MAA antigen, normally expressed by A375 melanoma cells in culture. Therefore, despite similar levels of tumour cell killing efficiencies *in vitro*, we observed improved efficacy with HMW-MAA specific IgE compared to the corresponding IgG1 of the same specificity *in vivo.* This result suggests that treatment with melanoma antigen-specific IgE antibody was superior in inducing tumour growth restriction *in vivo* compared to the corresponding IgG1.

The data presented in this chapter demonstrated that engineering antibodies with IgE-Fc regions can improve antibody effector functions, which is particularly advantageous in the case of solid tumours, since these are frequently refractory to treatment with IgG antibodies. With a serum half-life of 21-24 days, compared to a half-life of 2-3 days in

tissues, IgG antibodies may be the most effective antibody class to target blood-resident tumours and circulating tumour cells, while their ability to exert tumour surveillance in tissues may be less potent (Hellman, 2007; Ravetch & Kinet, 1991). Other parameters that may modulate IgG anti-tumoural functions could be slow or ineffective recruitment and/or local suppression of activator immune effector cells by tumour cells in lesions and the presence/induction of immunoregulatory cells by tumours *in situ* (Brigati et al, 2002). For antibodies of the IgG class that do localise in tumour lesions, overcoming these immunomodulatory environments may be challenging. Additionally, factors such as the low affinity of IgG for its Fc gamma receptors and the presence of the inhibitory receptor FcγRIIb in tumour-infiltrating immune cells such as macrophages may negatively influence the efficacy of IgG antibodies in tissues (Kraft & Kinet, 2007; Maenaka et al, 2001).

Since each antibody class operates in different anatomic compartments, and functions through unique Fc-receptors and immune effector cells, we have focused on antibodies of the IgE class, commonly known for their role in the allergic response and parasite protection. Antibodies of this class function through their specific high-affinity Fc receptors (FcεRl with $K_a=10^{11}$ M^{-1} is 10^2-10^5 times higher than that of IgGs for their receptors) on a different spectrum of effector cells to IgG, and naturally reside in tissues where they exert immunological surveillance. Our results demonstrate that these properties may translate to superior efficacy in targeting tissue-resident tumours such as melanoma.

In conclusion, antibodies of different classes may present different functional properties against cancer cells, due to their ability to activate different families of Fc receptors on

immune effector cells to destroy tumours. In the context of melanoma, the two chimaeric monoclonal antibodies IgG1 and IgE of the same specificity against HMW-MAA, which is over-expressed by more than 80% of melanomas, had differential effects *in vivo*. Due to differential immune effector cell-mediated melanoma tumour killing by each antibody, the IgE antibody had superior efficacy compared to the corresponding IgG1 in an *in vivo* xenograft model of melanoma. Future mouse models will further investigate the mechanisms by which these antibodies engage and activate different compartments of the immune response. Ultimately, this will help evaluate novel immunotherapeutic tools for the treatment of solid cancers, and will enrich our understanding of therapeutically-relevant antibody-mediated mechanisms of action against cancer cells. Furthermore, comparative SPECT imaging preclinical studies using radiolabeled IgE and IgG1 will investigate the efficacy of trafficking to and retention in tumour for each antibody class. The biology of IgE predicts superior biodistribution kinetics for this subclass, which could potentially be exploited in diagnosis as well as therapy.

6 DISCOVERY OF NOVEL ANTIBODIES AGAINST MELANOMA ANTIGENS

6.1 Introduction

Melanoma, highly aggressive and most lethal form of skin cancer affects people of all ages with incidence rates rising by 5% per year, faster than any other cancer in the UK (Karim-Kos et al, 2011). Currently, surgical excision remains the standard of care for the treatment of melanoma and yet about 20% of the primary melanomas progress to metastatic disease for which the treatment options are limited with a median survival of 8-18 months (Balch et al, 2009). Therefore, effective treatments are urgently needed for patients with this disease. Significant progress has been made in the treatment of advanced stage disease, with the FDA approval of Vemurafenib (BRAF V600E kinase inhibitor) and Ipilimumab (humanised mAb targeting the inhibitory molecule cytotoxic T-lymphocyte antigen 4) (Lacy et al, 2012). However, despite the partial success and promise of various immunotherapeutic strategies, including antibodies, there are presently no promising antibody therapies that directly target antigens on the surface of melanoma cells.

Immunising mice with specific human antigens, mainly in the form of tumour cell lysates, proteins or epitopes of tumour-associated proteins resulted in the generation of murine antibodies with tumour antigen-specific variable regions. These antibodies were produced using hybridoma technology *in vitro*, which allowed for sufficient amounts of antibodies to be produced for further investigation of their efficacy in cancer immunotherapy. However, the murine antibody is expected to induce HAMA (human anti-murine antibody) responses in patients, resulting in neutralisation of the antibody and rapid clearance from the circulation, thereby significantly reducing any efficacy

against tumour cells. In addition, the murine Fc region is not expected to effectively recruit FcR-expressing human immune effector cells which may target and kill tumour cells by mechanisms such as cytotoxicity and/or phagocytosis. This has resulted in the construction of chimaeric (murine variable and human constant regions), humanised (human framework and murine CDR/s) or fully human antibodies which do not induce an immune response (Jakobovits et al, 2007; Lanzavecchia et al, 2007; Winter & Harris, 1993) by recombinant DNA technologies. One of the most recent technologies is the use of human transgenic mice such as XenoMouse and VelocImmune (Jakobovits et al, 2007). These mice express human immunoglobulin gene loci and therefore, after immunisation with antigens, fully-human antibodies are secreted and subsequently produced by standard hybridoma technology *in vitro* (Jakobovits et al, 2007). Another technique Phage display, has been established for the discovery of fully-human antibodies by mimicking the natural immune process of antibody affinity maturation in an *in vitro* setting. A limitation of this technique, however, is that the antibodies produced must be matched with random light chains to obtain a fully affinity matured antibody (Winter & Harris, 1993).

Other methods for discovery of fully-human monoclonal antibodies derived from human B lymphocytes have also been exploited. B cells isolated from human blood, grown in culture and immortalised with Epstein Barr virus (EBV), have been activated with the toll-like receptor 9 (TLR-9) agonist CpG and screened against the SARS virus for selection of cells secreting antibodies specific for virus envelope proteins. Subsequently, 35 neutralising monoclonal antibodies against the SARS coronavirus from a convalescent patient have been generated (Lanzavecchia et al, 2006; Traggiai et al, 2004). Two years later, another group demonstrated that constant activation of B lymphocytes by exogenous stimuli (CD40L and IL-4) bears a close resemblance to

physiological T cell-driven clonal expansion, and is sufficient to conditionally immortalise these cells (Wiesner et al, 2008). The establishment of long- term antigen specific B cell lines by retroviral transduction of B lymphocytes with constructs expressing the Bcl-6 and Bcl-xL, proteins responsible for rescuing B cells from terminal differentiation into short-lived plasma cells in the germinal centers of lymphoid organs, has also been described (Diehl et al, 2008; Kwakkenbos et al, 2010).

The human monoclonal antibodies generated by majority of the above mentioned studies are highly specific against their antigens and are able to recruit the full immune response, thus representing a relatively quick identification approach for discovery of fully-human antibodies which in theory are naturally affinity-matured *in vivo*. Inspired by these studies, in the constant search for antibody therapies that directly target antigens on the surface of melanoma cells, fellow students at Karagiannis lab (Division of Genetics and Molecular Medicine, KCL) have developed novel methods for identification of antibodies against melanoma cancer antigens. A novel cell-based ELISA for screening of tumour cell-specific antibodies secreted by cultured patient-derived B cells was developed by Dr. Amy Gilbert (Gilbert et al, 2011). A direct single cell sorting technique using B cell markers and A375 melanoma cell line derived surface antigen markers (Figure 1.1) for isolation of cells secreting melanoma antigen-specific antibodies has been developed by Mr. Panos Karagiannis (manuscript in preparation)

Initially, B cells derived from cancer patients were screened for the production of tumour antigen-specific antibodies using the cell-based ELISA or single cell sorting in Karagiannis lab. My major contribution to this project included cDNA preparation from B cells clones or single sorted cells secreting antigen-specific antibodies, PCR amplification of antibody variable region genes using nested PCR reactions to create

natural antibody libraries, cloning and expression of recombinant IgG1 antibodies, confirmation of their ability to recognise tumour and effector cells and finally to test their potential therapeutic efficacy to target melanoma cells.

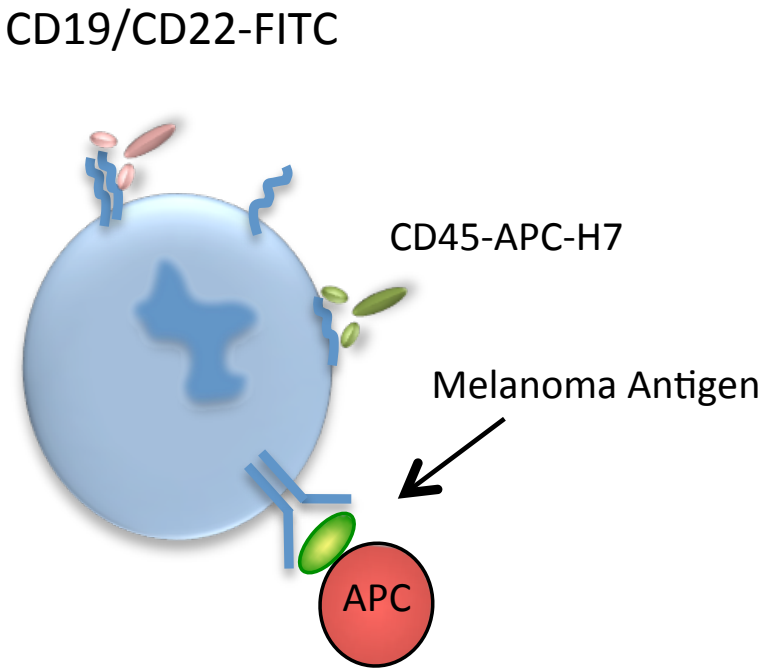


Figure 6.1: Schematic representation of single cell sorting. Single cell sorting of cancer patient derived B cells using CD19/CD22 B cell markers, CD45 leukocytes marker and A375 melanoma cell line derived surface antigen markers for isolation of cells secreting antibodies with specificity for melanoma antigens. Figure courtesy of Panos Karagiannis (PhD student, KCL).

6.2 Methods

6.2.1 cDNA preparation

Cultured patient-derived B cell clones were pelleted by centrifugation. The supernatant was completely removed by aspiration and the pellet was resuspended in RLT lysis buffer (Qiagen) containing 10% β-mercaptoethanol. Cells were homogenised by briefly pulsing with a hand held cordless motor homogeniser (Anachem). RNA was isolated from the homogenate using Qiagen's RNeasy Protect Mini Kit following the manufacturer's instructions and eluted from the columns in 30µl RNase free water. 1µg RNA was reverse transcribed to cDNA using 400U M-MLV Reverse Transcriptase (Invitrogen), 0.05ng random primers, 0.2ng oligo dT primers (both Promega), 0.4mM dNTPs and 8U RNase OUT (Invitrogen) in 1 x 1st strand buffer (Invitrogen) and 5mM DTT (Invitrogen). The reverse transcription reaction was incubated at 37°C for 10 minutes, 42°C for 45 minutes then 50°C for 10 minutes. The production of cDNA was analysed by a PCR reaction to detect the housekeeping enzyme GAPDH gene. 20 µl of PCR reaction per sample were prepared using Phusion^{TM} Flash High-Fidelity PCR Master Mix (Finnzymes) containing 0.5µM GAPDH primers (Table 2.2) and 2 µl cDNA. Reaction mixtures were denatured at 98°C for 1 minute followed by 35 thermal cycles of: 98°C for 10 seconds; 60°C for 30 seconds; and 72°C for 15 seconds. PCR products were analysed by gel electrophoresis on 1.5% agarose gel (Figure 6.2). The gel electrophoresis showed single bands, indicating amplified GAPDH cDNA (~450 bp), for single sorted B cell M394 and B cell clones M80-F2 and M80-F4, which were selected for further assessments.

6.2.2 Cloning IgG1 constructs

Following the primer design steps outlined in Figure 4.5, I have designed primer LinearG1_Fwd (Table 2.2) to work in conjunction with previously designed Linear_Krev and primer pair Linear_Kfwd and Linear_Hrev (Figure 6.4). Thus, expression vector pVitroMAA-IgG1 was linearised by primer pair Linear_Kfwd and Linear_Hrev, and primer pair LinearG1_Fwd and Linear_Krev in two independent PCR reactions as previously described (Section 4.5). Simultaneously, the sequenced V_H and V_K nested PCR products of M394, M80-F2 and M80-F4 clones were amplified by primer pairs M394H_Fwd and M394H_Rev, M394K_Fwd and M394K_Rev, M80-F2H_Fwd and M80-F2H_Rev, M80-F2K_Fwd and M80-F2K_Rev, M80-F4H_Fwd and M80-F4H_Rev, M80-F4K_Fwd and M80-F4K_Rev respectively (Table 2.2) for generation of vector fragment terminal end-homology as previously described (Section 4.5). The *Dpn I* treated vector fragments were mixed with unpurified V_H and V_K PCR products in 1:1:1:1 (v/v) ratio and 2 μl from the mixture transformed into XL1-Blue competent cells as previously described (Methods 4.2)

6.3 Results

6.3.1 cDNA synthesis and amplification of antibody variable region genes from patient derived B cells

To isolate cDNA, single sorted B cells and cultured patient-derived B cell clones from Karagiannis lab were treated differently. cDNA from single sorted B cells was prepared as previously described (James et al, 2012). The antibody variable regions were amplified by two rounds of nested PCRs using nested set of V_H, Vκ and Vλ primers designed to cover all possible human V genes as previously described (James et al, 2012)

The amplified PCR products were analysed by gel electrophoresis on 1.5% agarose gel (Figure 6.3). The gel electrophoresis showed amplification of V_H and V_L in all three clones and the PCR products were sent for DNA sequencing to confirm their identity. DNA sequencing result confirmed three sets of V_H and V_L genes, which were subsequently analysed on BLAST 2.0 (http://blast.ncbi.nlm.nih.gov/) to conform sequence homology to other published sequences of human IgG. Further analysis of clonal families and rearrangement was performed using IMGT/V-Quest (http://www.imgt.org/IMGT_vquest/share/textes). The analyses showed that the variable region DNA M80-F2 clone belonged to VH3 family with kappa light chain antibody, variable region from M80-F4 clone belonged to VH4 family with kappa light chain and clone M394 antibody variable region matched to VH3 family with kappa light chain antibody (data not shown).

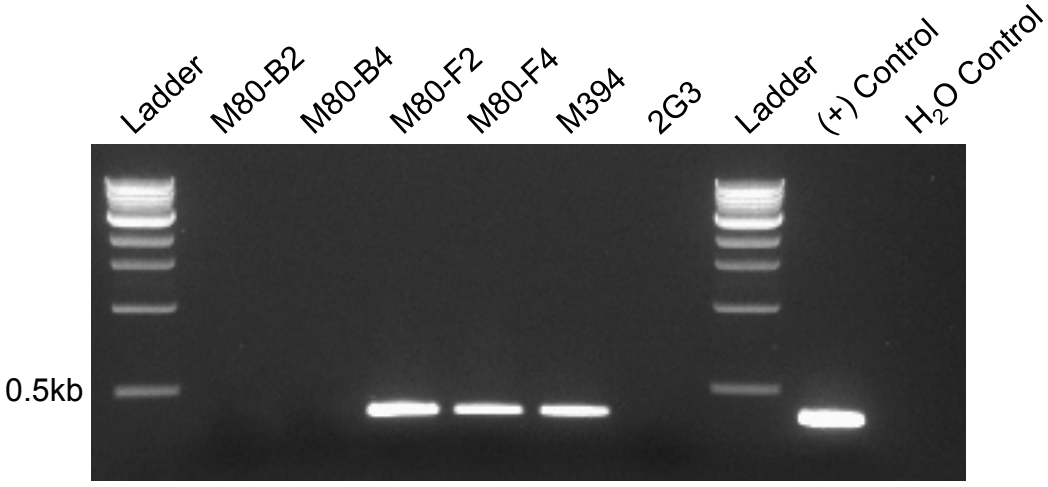


Figure 6.2: Amplification of GAPDH from patient derived cDNA. The housekeeping enzyme GAPDH gene (~450bp) was used to verify cDNA sample preparation from cancer patient derived single sorted B cells (M394) or B cell clones (M80-B2, M80-B4, M80-F2, M80-F4 and 2G3), alongside 1kb DNA Ladder (NEB) and HEK293 (+) control.

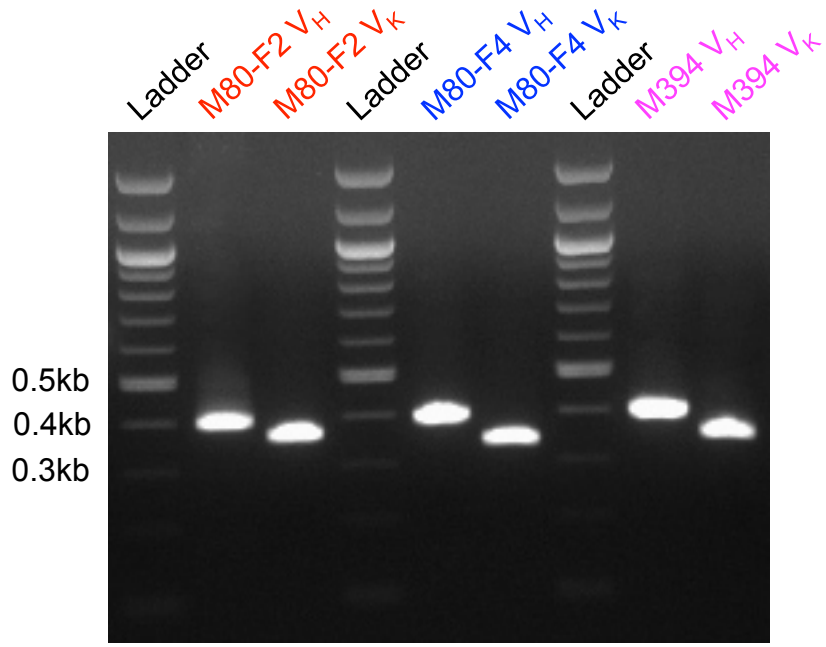


Figure 6.3: Amplification of V_H and V_L genes from patient derived cDNA. V_H and V_L gene transcripts were RT-PCR amplified from cancer patient derived single sorted B cell (M394) or B cell clones (M80-F2 and M80-F4) cDNA using nested forward primers specific to each V_H and V_L family and nested reverse primers specific for the first domain of the IgG, Igκ and Igλ constant region, alongside 100bp DNA Ladder (NEB).

6.3.2 Generation of IgG1 constructs with patient derived variable regions

As mentioned in chapter 4, I have successfully utilised the PIPE cloning method for swapping variable regions in IgE expression constructs. Our antibody discovery program provided me with an ideal opportunity to test the method and support the program by generating IgG1 constructs with patient derived variable regions. DNA sequencing of single colonies confirmed the correct assembly of the vector fragments with the patient derived V_H and V_K regions by the PIPE cloning reaction and the newly generated vectors were named pVitroM80-F2-IgG1, pVitroM80-F4-IgG1 and pVitroM394-IgG1. This cloning experiment once again confirmed the seamless exchange of the specificity of an antibody by the PIPE cloning method, and potential capability of supporting the high-throughput screening of antibody candidates derived from antibody discovery programs.

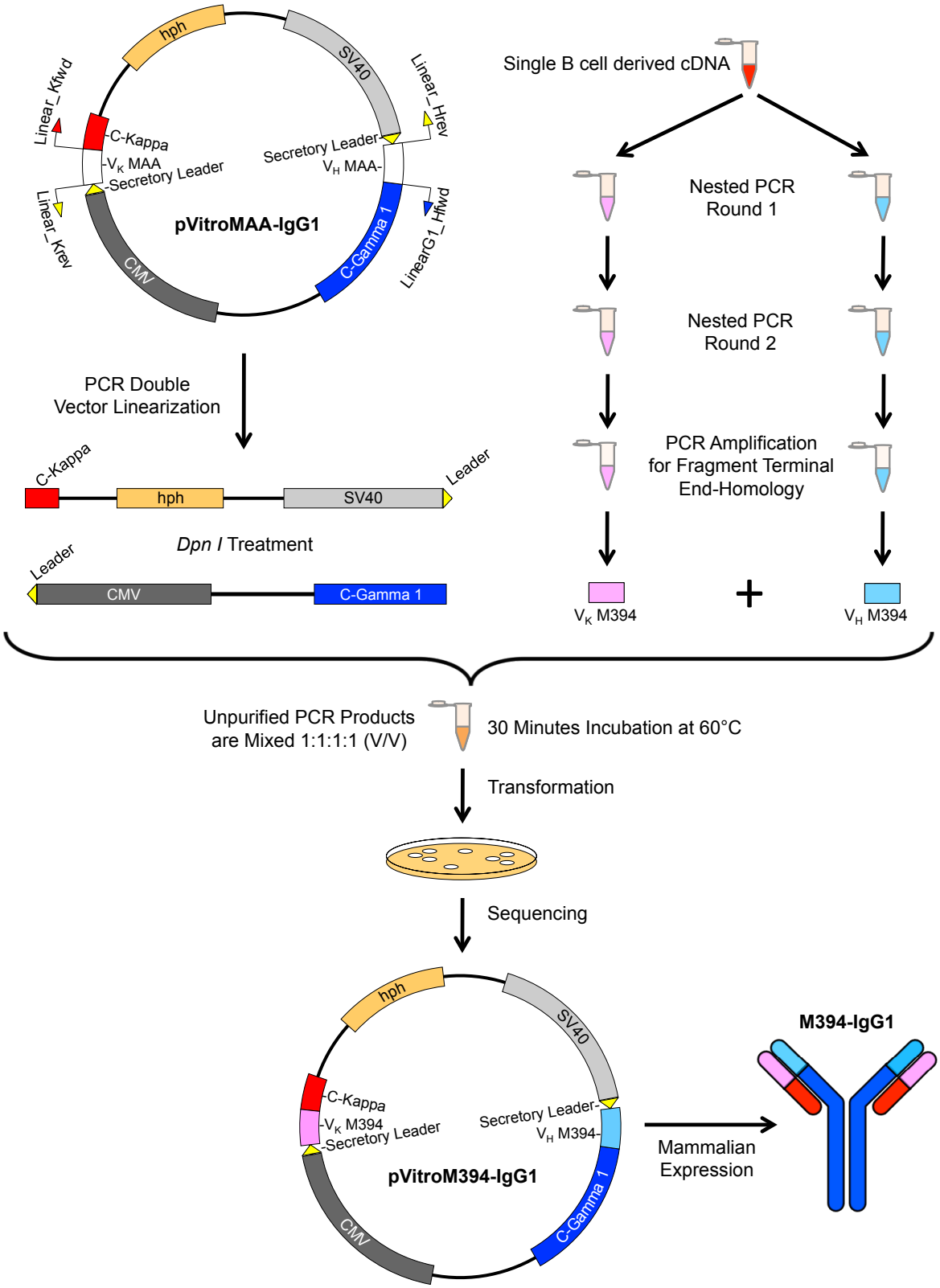


Figure 6.4: Schematic representation of B cell derived V genes swapping cloning procedure. pVitroMAA-IgG1 vector is linearised by two V_H and V_K flanking primer pairs, resulting in two vector fragments subsequently treated by *Dpn I*. Simultaneously, V_H and V_L genes transcripts are RT-PCR amplified from patient derived cDNA using nested sets of primers and subsequently amplified for generation of vector fragment terminal end-homology. The *Dpn I* treated vector fragments are mixed 1:1:1:1 (v/v) with unpurified M394 V_H and V_K. The single-stranded DNA fragments anneal directionally across the complementary sequences and nicks and gaps are repaired *in vivo* after transformation, generating pVitroM394-IgG1 expression vector.

6.3.3 Production and characterisation of IgG1 antibodies with patient derived variable regions

Having successfully cloned pVitroM80-F2-IgG1, pVitroM80-F4-IgG1 and pVitroM394-IgG1 vectors, I continued with the expression of the IgG1 antibodies. 293-F cells were transfected as previously described (Methods 4.2). The cells were kept under Hygromycin selection for 2 weeks and stable cell lines were generated. The IgG1 expressing cell lines were expanded into 1L shaker flasks and sample supernatants collected after 14 days for antibody quantification by anti-human IgG1 ELISA as described previously (Gilbert et al, 2011; McCloskey et al, 2007). The ELISA analysis showed 28mg/L M80-F2 IgG1, 20mg/L M80-F4 IgG1 and 18mg/L M394 IgG1 expression levels (Figure 6.5). This result further confirmed that the stable antibody expression system was capable of generating material in quantities that could support animal model experiments, enabling me to continue with the purification process.

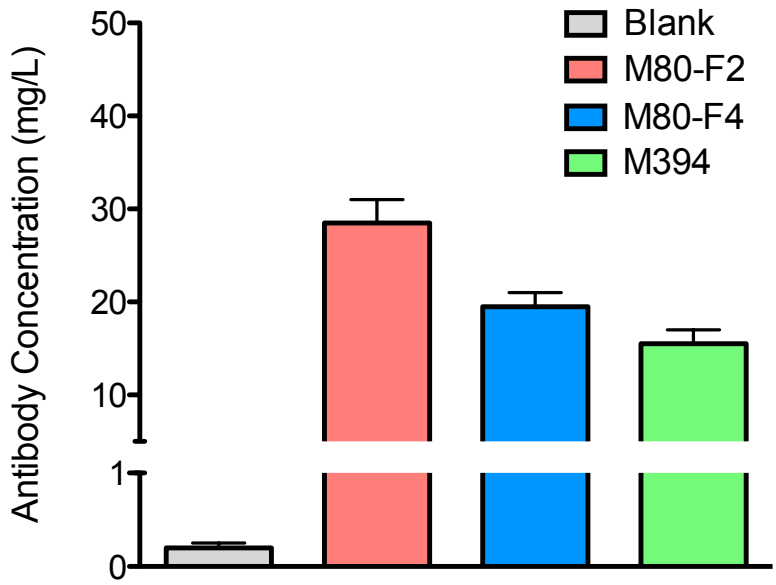


Figure 6.5: Stable expression of B cell derived IgG1 antibodies. Anti-human IgG1 ELISA analysis of supernatants from recombinantly expressed B cell derived clones cultured in 1L spinners. The antibody concentration at mg/L was determined by reference to a standard curve and the results represent the mean of triplicate readings ± SD.

The IgG1 antibodies were purified on a Protein-G column as previously described (Methods 5.2). The affinity column-purified products were analysed by size-exclusion chromatography (Hunt et al, 2005) together with isotype control MOv18 IgG1 (Karagiannis et al, 2003). The size-exclusion chromatography analysis showed no aggregation and confirmed the affinity column-purified product consists of monomeric antibodies (Figure 6.6). The size and purity of affinity column-purified IgG1 antibodies, were assessed by SDS-PAGE analysis as previously described (Methods 4.2). Under non-reducing conditions (Figure 6.7), molecular sizes of the visualised protein bands were found to be in the range of 148-250 kDa of the protein ladder representing the heterotetrameric IgG1 antibody. Free light or heavy chain was not detected, suggesting that the antibody chains are assembled into whole antibody molecules. Thus, the represented molecular sizes in SDS–PAGE suggest that the secreted antibodies are properly folded and glycosylated.

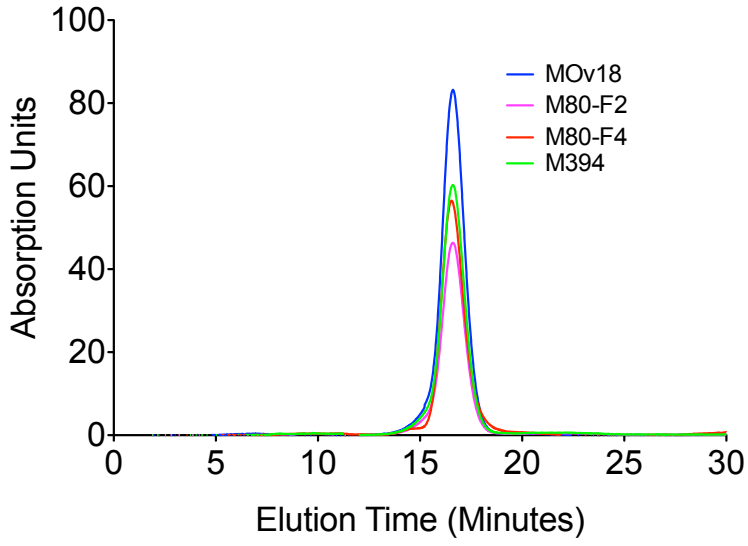


Figure 6.6: Size exclusion chromatography analysis of B cell derived IgG1 antibodies. Elution profiles of recombinantly expressed cancer patient derived IgG1 antibodies, compared to previously-characterised isotype control MOv18 IgG1, from Superdex™ 200 gel filtration. The antibody profiles correspond to isotype control and confirm the affinity purified products consist of monomeric antibodies.

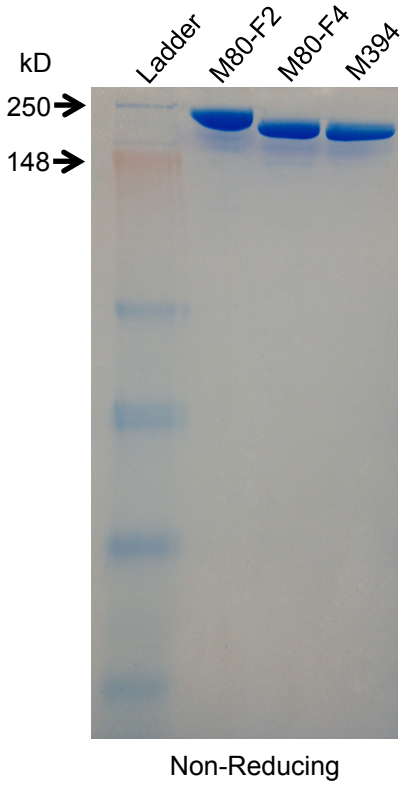


Figure 6.7: SDS-PAGE analysis of B cell derived IgG1 antibodies. Affinity purified recombinantly expressed cancer patient derived IgG1 antibodies were analysed under non-reducing conditions, alongside SeeBlue® Plus2 Pre-Stained Standard, visualised by Coomassie staining. The represented antibody molecular masses show the secreted antibodies are properly folded and glycosylated.

6.3.4 Flow cytometric assessment of IgG1 antibodies with patient derived variable regions

The reactivity of the affinity column-purified recombinantly expressed IgG1 antibodies, with patient derived variable regions, against melanoma cells was analysed by flow cytometry. The flow cytometric assessments were used to compare specificity of M80-F2, M80-F4 and M394 IgG1 clones to native antigens on the cell surface of A375 melanoma cells, compared to previously characterised HMW-MAA specific IgG1 (Chapter 5).

Flow cytometric analysis (Figure 6.8) of A375 melanoma cells incubated with M80-F2 and M80-F4 IgG1 antibodies, did not show a clear shift of fluorescence intensity, suggesting that these clones do not have specificity for melanoma antigens. However, M394 IgG1 clone had a small but noticeable shift of fluorescence intensity, compared to 100% binding of HMW-MAA specific IgG1, suggesting potential specificity for

melanoma antigens as presented on the cell surface of A375 cells. Therefore, the M394 IgG1 clone was subjected to further flow cytometric assessments using primary melanoma cells and cell lines from skin, lung and lymph metastases and primary keratinocytes and melanocytes.

Flow cytometric analysis (Figure 6.9) of cell lines from skin metastases (A375 and G-361), lung metastasis (Malme-3M), lymph metastasis (A2058) and primary melanoma (Wm-115) cells showed specific binding of M394 IgG1 clone to melanoma cells and no binding above background to primary keratinocytes and melanocytes. Furthermore, the IgG1 bound to the surface of U937 cells (data not shown), expressing Fcγ receptors, demonstrating functional receptor-binding activity of the recombinant antibody.

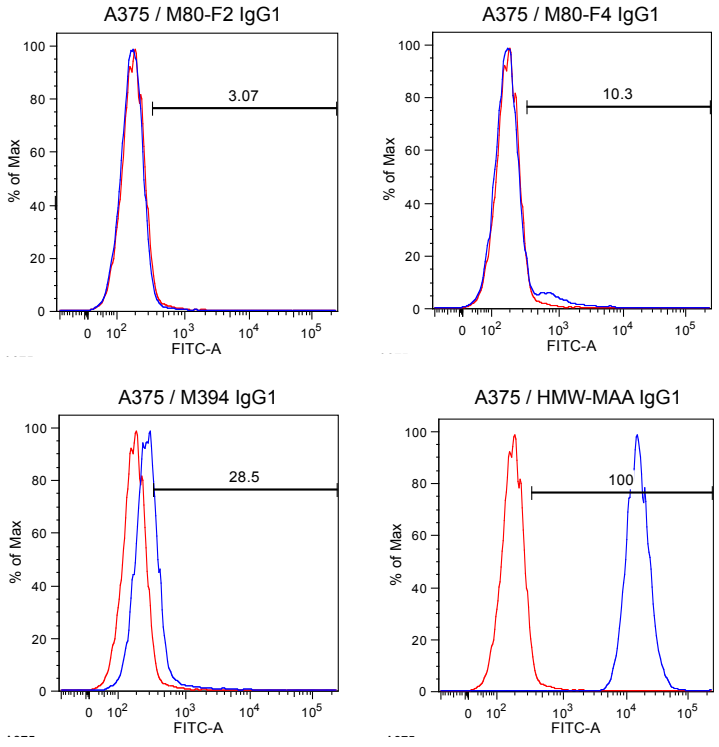


Figure 6.8: Flow cytometric assessment of B cell derived IgG1 antibodies. Recombinantly expressed cancer patient derived B cell clones M80-F2 and M80-F4 do not bind to the cell surface of A375 melanoma cells. The single sorted B cell derived M394 recombinant antibody shows weak binding to A375 cells, compared to 100% binding of previously characterised HMW-MAA specific IgG1. Antibody binding was detected using a goat anti-human IgG-FITC antibody.

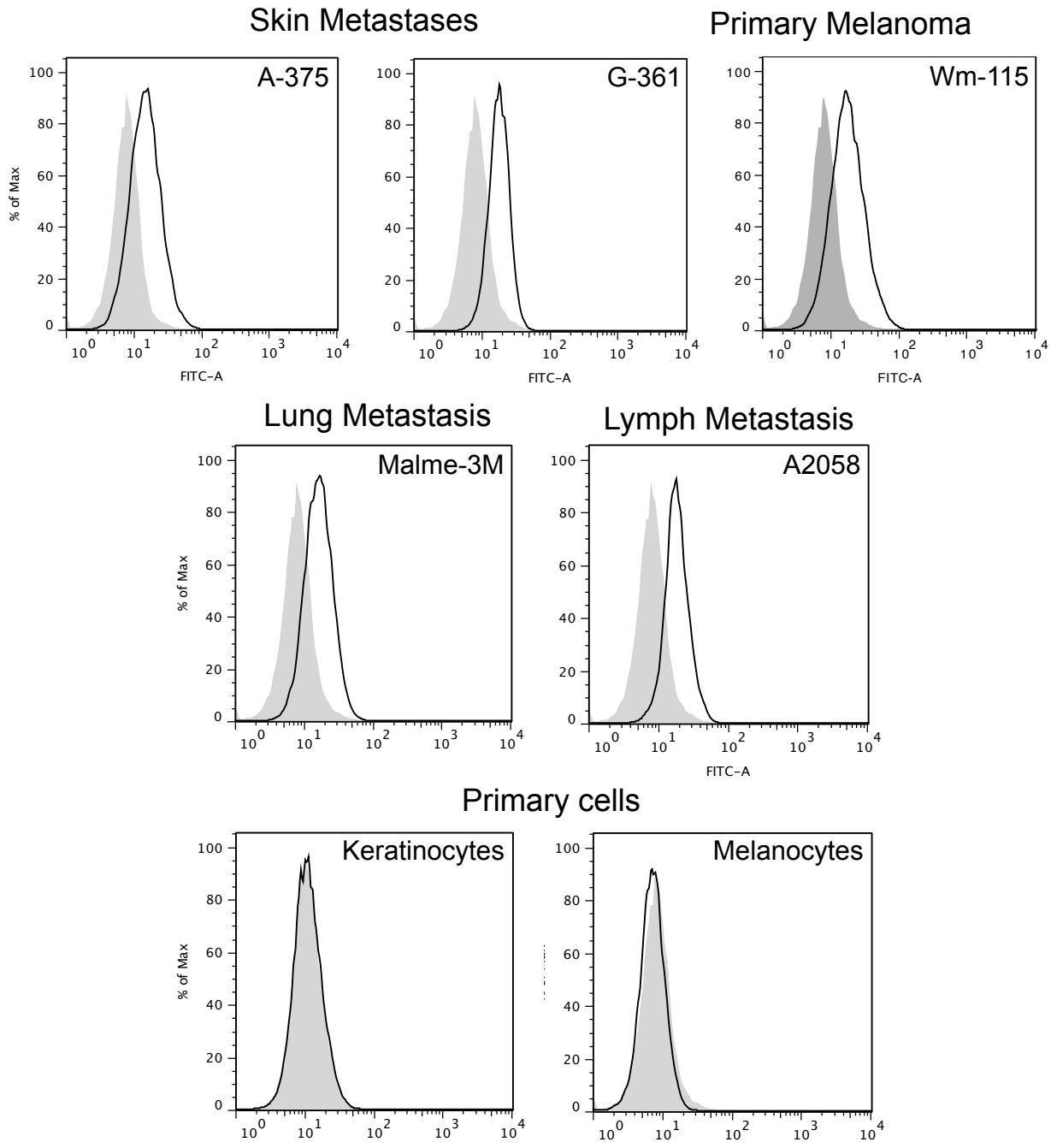


Figure 6.9: Flow cytometric assessment of M394 IgG1 clone. The single sorted B cell derived recombinantly expressed M394 IgG1 antibody shows binding to primary melanoma cells and cell lines from skin, lung and lymph metastases and no binding above background to primary keratinocytes and melanocytes. Antibody binding was detected using a goat anti-human IgG-FITC antibody. Figure courtesy of Panos Karagiannis (PhD student, KCL).

6.3.5 Flow cytometric ADCC/ ADCP assessment of M394 IgG1

Having successfully demonstrated that the recombinantly expressed M394 IgG1 clone shows specific binding to melanoma as well as immune effector cells by flow cytometry, I aimed to examine the ability of M394 IgG1 to directly signal tumour cell death, measured by tumour cell cytotoxicity (ADCC) and phagocytosis (ADCP) of A375 melanoma cells as previously described (Section 5.6)

The three-color flow cytometric assay showed that after 3 hours in culture, U937 monocytic cells, employed as effector cells, mixed with M394 IgG1 and A375 did not mediate significant levels of ADCP above that seen with samples incubated with the unspecific isotype control MOv18 IgG1 (Figure 6.10). However, the antibody mediated predominantly ADCC with a 2-fold increase above that seen with samples incubated with the HMW-MAA specific IgG1, an antibody previously shown to kill melanoma cells by ADCP (Section 5.6). Thus, the flow cytometric ADCC/ADCP assay measurements confirmed that the recombinantly expressed M394 IgG1 antibody mediated significant levels of ADCC of A375 tumour cells by monocytic cells, indicating a potential clinical application for the treatment of melanoma.

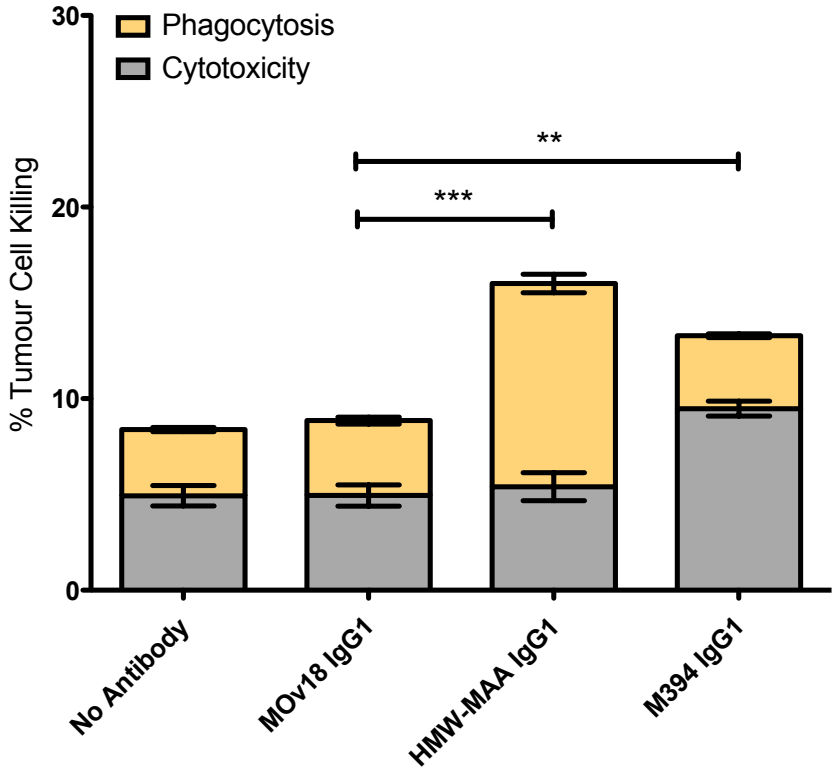


Figure 6.10: Three-colour flow cytometric tumour cell killing assessment of M394 IgG1. Quantification of M394 IgG1-mediated A375 tumour cell killing by U937 monocytes is measured by ADCC/ ADCP assay. The single sorted B cell derived M394 recombinant antibody induces tumor cell killing via cytotoxicity. Cytotoxicity: grey bars; phagocytosis: gold bars. Results are means ± SD of three independent experiments. (n=5; *p<0.05; **p<0.01; ***p<0.001; ns: p>0.05). Figure courtesy of Panos Karagiannis (PhD student, KCL).

6.4 Discussion

The most lethal and highly aggressive form of skin cancer, melanoma, affects people of all ages with incidence rates rising every year, faster than any other cancer in the UK (Karim-Kos et al, 2011). Surgical excision remains the standard of care for the treatment of melanoma and yet about 20% of the primary melanomas progress to metastatic disease for which the treatment options are limited (Balch et al, 2009). Despite the partial success and promise of various immunotherapeutic strategies, including antibodies (Lacy et al, 2012), there are presently no promising antibody therapies that directly target antigens on the surface of melanoma cells and effective treatments are urgently needed for patients with this disease.

Tumour antigen-specific antibodies have been generated using hybridoma technology *in vitro*, in sufficient amounts for further investigations of their efficacy in cancer immunotherapy. However, the murine antibody is likely to induce human anti-murine antibody responses in patients, resulting in neutralisation of the antibody and rapid clearance from the circulation, reducing the efficacy against tumour cells. In addition, the murine Fc region is not expected to effectively recruit FcR-expressing human immune effector cells which may target and kill tumour cells by mechanisms such as cytotoxicity and/or phagocytosis. Recombinant DNA technologies have been developed to tackle these limitations by constructing chimaeric, humanised or fully human antibodies which do not induce an immune response (Jakobovits et al, 2007; Lanzavecchia et al, 2007; Winter & Harris, 1993). Another technique Phage display, has been established for the discovery of fully-human antibodies with the limitation of matching the antigen specific heavy chain with random light chains to obtain a fully affinity matured antibody (Winter & Harris, 1993). Fully human monoclonal antibodies, highly specific against their antigens, have also been discovered by EBV immortalised human B lymphocytes (Lanzavecchia et al, 2006; Traggiai et al, 2004).

Taking advantage of these studies, a novel cell-based ELISA for screening of tumour cell-specific antibodies secreted by cultured patient derived B cells (Gilbert et al, 2011) and a direct single cell sorting technique using B cell markers and A375 melanoma cell line derived surface antigen markers (Figure 1.1) for isolation of cells secreting antibodies with specificity for melanoma antigens have been developed in Karagiannis lab. Single sorted B cells and cultured patient derived B cell clones from Karagiannis lab were used for cDNA preparation and nested PCR amplification of antibody variable regions as previously described (James et al, 2012). Gel electrophoresis of the amplified PCR products showed bands representing three sets of V_H and V_L genes for one single sorted B cell clone (M394) and two cultured patient derived B cell clones (M80-F2 and

M80-F4) respectively. The V_H and V_L genes were subsequently confirmed by DNA sequencing and analysis of clonal families and rearrangement was performed using IMGT/V-Quest. The analysis showed all three clones possessed kappa light chain and revealed the M80-F2 and M394 clones belonged to VH3 family, whilst M80-F4 was derived from VH4 family.

Having successfully adapted and optimised the PIPE cloning method for swapping variable regions in chapter 4, I aimed to exchange the HMW-MAA specific V_H and V_K in vector pVitroMAA-IgG1 with the melanoma patient derived variables. Following an additional primer design, specific to human Cγ1, and previously described cloning procedure, vectors pVitroM80-F2-IgG1, pVitroM80-F4-IgG1 and pVitroM394-IgG1 were generated in a single day. This cloning experiment confirmed the seamless exchange of the specificity of an antibody by the PIPE cloning method, capable of supporting the high-throughput screening of antibody candidates derived from antibody discovery programs.

Having successfully cloned the patient derived antibody variable regions into pVITRO IgG1 expressing vector, stable 293-F cell lines were generated by a small-scale transfection and 2 weeks selection with Hygromycin B. Following cell line expansion into 1L shaker flasks and 14 days incubation, the antibody expression yields were quantified by anti-human IgG1 ELISA. The ELISA analysis showed 28mg/L M80-F2 IgG1, 20mg/L M80-F4 IgG1 and 18mg/L M394 IgG1 expression levels, confirming the stable antibody expression system was capable of generating material in quantities that could support animal model experiments, enabling me to continue with the purification process.

The IgG1 antibodies were purified on a Protein-G column and the affinity column-purified products were analysed by size-exclusion chromatography. The analysis showed no aggregation and confirmed the affinity column-purified product consists of monomeric antibodies. The size and purity of affinity column-purified IgG1 antibodies, were assessed by SDS-PAGE analysis. Under non-reducing conditions, free light or heavy chain was not detected, indicating the antibody chains are assembled into whole antibody molecules and that the affinity purification was effective in yielding pure antibodies. The represented molecular sizes in SDS–PAGE suggested that the secreted antibodies are properly folded and glycosylated. These results represent the effectiveness of our antibody cloning system in swapping variable regions, simplifying the subsequent antibody production in sufficient quantities, capable of supporting the parallel characterisation and comparative functional studies between different melanoma patient derived antibodies with clinical potential.

Biological reactivity of the affinity column-purified recombinantly expressed IgG1 antibodies, with patient derived variable regions, against melanoma cells was analysed by flow cytometry. The flow cytometric analysis of A375 melanoma cells incubated with the two cultured patient derived B cell clones (M80-F2 and M80-F4), show no binding, suggesting that these clones were not specific for melanoma antigens. However, the single sorted B cell clone (M394) showed a weak binding compared to 100% binding of HMW-MAA specific IgG1, suggesting potential specificity for melanoma antigens as presented on the cell surface of A375 cells. In subsequent flow cytometric experiments, the M394 IgG1 clone showed specific binding to skin, lung and lymph metastasis and primary melanoma cells and no binding above background to primary keratinocytes and melanocytes. Furthermore, the IgG1 bound to the surface of

U937 cells, expressing Fcγ receptors, demonstrating functional receptor-binding activity of the recombinant antibody.

Finally, to assess the ability of M394 IgG1 clone to directly signal tumour cell death, the tumour cell cytotoxicity (ADCC) and phagocytosis (ADCP) of A375 melanoma cells, were measured where U937 monocytic cells were employed as effector cells. The three-color flow cytometric assay showed that after 3 hours in culture, the M394 IgG1 was capable of activating immune effector cells to kill cancer cells *in vitro,* mediating predominantly ADCC, indicating a potential clinical application for the treatment of melanoma. We have shown that the HMW-MAA specific IgE antibody had superior efficacy compared to the corresponding IgG1 in an *in vivo* xenograft model of melanoma. Future cloning of M394 as an IgE antibody, will enable the comparative functional studies between IgG1 and IgE isotypes in mouse models, dissecting the mechanisms by which these antibodies engage and activate different compartments of the immune response.

In conclusion, we have developed an efficient method using single cell sorting of melanoma patient derived B cells to discover antibodies specific for melanoma antigens. The patient derived antibody variable regions were seamlessly integrated into IgG1 expression construct using our antibody cloning system, which proved to be very effective, readily adaptable for the high-throughput screening of antibody candidates, simplifying the subsequent antibody production in quantities, capable of supporting the parallel characterisation and functional studies of a number of antibodies with clinical potential. We discovered an antibody clone (M394) that specifically binds melanoma cells, and is capable of tumour cell killing via ADCC in an *in vitro* assay, encouraging us to continue developing the antibody discovery method and pursuing melanoma specific antibodies for future effective antibody therapies of melanoma.

7 FINAL DISCUSSION

Over 100 years since the discovery of the "magic bullet", recombinant monoclonal antibodies, capable of recognising millions of antigens and executing different effector functions depending on the isotype, have become a key tool for basic research, diagnosis and treatment of human diseases. The increasing demand for therapeutic antibodies has resulted in a significant improvement in antibody production systems. However, the lack of a suitable manufacturing platform, which ensures consistent antibody production, has always been one of the major impediments to the development of recombinant antibody material in academia.

Conventional methods for recombinant expression of antibodies such as the establishment of cell lines tend to be a lengthy, low efficiency process involving extensive selection and screening and is consequently unfavourable for the rapid production of large numbers of antibodies for functional studies, as may be required after generating a variable gene library. To tackle this, tailoring the antibody expression cassettes for use in a vector suitable for transient transfection in mammalian HEK293E cells has enabled rapid antibody production (Baldi et al, 2005; Berntzen et al, 2005; Li et al, 2007; Wright et al, 2003). Furthermore, antibody variable region genes isolated from single cells have been integrated into human IgG1 expression constructs. However, the restriction sites used in these vectors are not compatible with all human constant gene isotypes.

To fulfil these limitations, our lab had previsouly designed and constructed a set of cloning cassettes for antibody heavy chain, as well as kappa and lambda light chains in which restrictions sites, compatible with all human C gene isotypes and all human and murine germline V genes, flanking the V and C domains have been introduced by silent

mutation (Figure 3.2). This has enabled the production of any conceivable human or mouse-human chimeric antibody by a transient expression system (Gan, 2008). I have used this system as a model for my current studies and as a standard for further optimisations. After successfully achieving previously reported expression levels (Gan, 2008), I continued with the system optimisation for better yields. Important parameters including transfecting agent affecting cell toxicity (Godbey et al, 2000), light : heavy chain ratio influencing Ig production (Baldi et al, 2005; Schlatter et al, 2005) and Tryptone N1 (Pham et al, 2005) or Optimab (Invitrogen) media supplementation were investigated. Most favourable cell viability and maximum antibody expression level was achieved at 4μg PEI : 1μg DNA ratio and 2:1 light : heavy chain DNA ratio. This optimisation halved the DNA amount of the light chain previously used (4:1) (Gan, 2008) to transfect cells, hence shortening the DNA production process required for transfection. The Tryptone N1 media supplementation had a 4-fold decrease in antibody expression level, compared to media alone, suggesting the peptone depletion was not limiting antibody production yield in HEK293E cells. Although the Optimab supplementation represented a 2-fold increase in volumetric antibody productivity, soon after the optimisation step was conducted, Invitrogen discontinued Optimab and no appropriate alternatives have been found so far.

Utilising the new optimised conditions, I swapped existing antibody variable regions with those specific for a High Molecular Weight Melanoma-Associated Antigen (HMW-MAA) within pSG vectors (Figure 3.11) and produced a fully functional antibody using the transient expression system. Following construct generation, using the optimised protocol, the HMW-MAA specific IgE antibody was transiently expressed and its antigen specificity and receptor-binding activity confirmed by flow cytometry.

Different vectors hosting a dual antibody expression cassette have been exploited and shown to produce higher antibody expression levels (Braren et al, 2007; Hecker et al, 2011; Wiberg et al, 2006). To eliminate the requirement for co-transfection of two vectors, coding for the expression of heavy and light antibody chains independently, I constructed a dual expression cassette in a single mammalian expression vector (Figure 4.6) thus halving the time and effort needed for DNA preparation and transfection procedure. I took advantage of the Polymerase Incomplete Primer Extension (PIPE) cloning method, shown to be rapid, cost-effective, and highly efficient, and thus capable of supporting the high-throughput cloning of thousands of genes in parallel (Klock et al, 2008), without the use of restriction enzymes- and ligation-dependent cloning. Having successfully constructed the dual cassette, I designed universal primer pairs flanking the variable regions and exchanged the specificity of an IgE antibody in a single day (Figure 4.7). This system enables high-throughput screening of antibody candidates derived from antibody discovery programs. Moreover, the construct codes for the expression of different antibody isotypes with the same antigen specificity, and thus supports the parallel characterisation and comparative functional studies between different isotypes. For example, I was able to successfully exchange the isotype of a HMW-MAA specific IgE to both IgG4 and IgG1 (Figure 4.9 and Figure 5.1). These cloning experiments represent the system's simplicity to exchange variable- and/or constant-region antibody domains allowing cloning of different isotypes with any desired specificity.

Due to the presence of a Hygromycin resistant gene in pVITRO1 vector, it was possible to perform a small-scale transfection, followed by 2 weeks selection and generation of stable cell lines. This enabled the subsequent expansion and large-scale production of

antibodies to support *in vivo* animal model experiments. Following affinity column purification, the recombinantly produced antibodies were assessed by SDS-PAGE, size-exclusion chromatography and flow cytometric analysis. The analysis demonstrated properly folded and glycosylated product, consisting of monomeric antibodies, capable of specifically recognising the target antigen, with functional receptor-binding activity.

Having successfully optimised the cloning and expression system for large-scale production of fully functional antibodies, I implemented it for parallel comparative functional studies between two different isotypes with the same specificity using validated *in vitro* and *ex vivo* assays. Tumour antigen-specific monoclonal IgG antibodies have already been approved for therapeutic use, but nearly all antibody therapies developed to date rely on IgG1 isotype (Scott et al, 2012). However, it is known that IgG diffuses inefficiently into solid tissues, and binds with low affinity to receptors on immune effector cells, while IgE antibodies can be transported from the circulation into tissues, where, through the strong affinity for their receptors on immune cells, they are known to trigger powerful immune responses. We have found that the results of *in vitro* experiments using IgG1 do not translate well to the solid tumour environment, where other mechanisms may prevail (Bracher et al, 2007; Karagiannis et al, 2008b; Karagiannis et al, 2007; Reali et al, 2001). We have demonstrated superior *in vivo* efficacy for the IgE subclass in comparison to IgG in the targeting of MOv18, an antibody directed against folate receptor, a tumour- specific antigen, in two murine xenograft models of human ovarian cancer (Gould et al, 1999; Karagiannis et al, 2003). Following successful characterisation and confirmation of the specific antigen recognition and functional receptor-binding activity by flow cytometry and immunofluorescence experiments, we examined the capacity of the HMW-MAA specific IgG1 and IgE antibodies to target and kill melanoma tumour cells. In a

functional degranulation assay measuring % β-hexosaminidase release by RBL SX-38 mast cells expressing human FcεRI, the IgE isotype demonstrated its ability to activate immune effector cells through engagement of its high affinity receptor. This isotype was also capable of activating immune effector cells to kill cancer cells *in vitro* by ADCC. Furthermore, treatment with melanoma antigen-specific IgE antibody was superior in inducing tumour growth restriction *in vivo* compared to the corresponding IgG1. This data suggests that engineering antibodies with IgE-Fc regions can improve antibody effector functions, which is particularly advantageous in the case of solid tumours, since these are frequently refractory to treatment with IgG antibodies. With a serum half-life of 21-24 days, compared to a half-life of 2-3 days in tissues, IgG antibodies may be the most effective antibody class to target blood-resident tumours and circulating tumour cells, while their ability to exert tumour surveillance in tissues may be less potent (Hellman, 2007; Ravetch & Kinet, 1991). The presence of the inhibitory receptor FcγRIIb in tumour-infiltrating immune cells such as macrophages may also negatively influence the efficacy of IgG antibodies in tissues (Kraft & Kinet, 2007; Maenaka et al, 2001). On the contrary, the IgE antibodies function through their specific high-affinity Fc receptors (FcεRl with $K_a=10^{11}$ M^{-1} is 10^2-10^5 times higher than that of IgGs for their receptors) on a different spectrum of effector cells to IgG, and naturally reside in tissues where they exert immunological surveillance. Our results demonstrate that these properties may translate to superior efficacy in targeting tissue-resident tumours such as melanoma. Future mouse models will further investigate the mechanisms by which these antibodies engage and activate different compartments of the immune response to help evaluate novel immunotherapeutic tools for the treatment of solid cancers. Evaluating the potential clinical use of IgE for the treatment of solid tumours will help translate this isotype from the laboratory bench to the clinic.

Having demonstrated the anti-tumour activity of a recombinantly produced chimeric antibody specific for a known melanoma antigen (HMW-MAA), we had a golden standard to use as a control for the evaluation of patient-derived fully-human melanoma antigen-specific antibodies. To support the third aim of the antibody discovery program, patient-derived single sorted B cells from Karagiannis lab were used for cDNA preparation and antibody variable regions were amplified by a nested PCR reaction. DNA sequencing confirmed variable gene pairs were seamlessly cloned into IgG1 expression vectors and produced using the stable antibody expression system. Flow cytometric experiments of a recombinantly expressed IgG1 antibody, with patient derived variable regions, showed specific binding to cell lines from skin, lung and lymph metastasis and primary melanoma cells and no binding above background to primary keratinocytes and melanocytes. Furthermore, the antibody was capable of activating immune effector cells to kill cancer cells *in vitro,* mediating predominantly ADCC, encouraging us to continue developing the antibody discovery method and pursuing melanoma antigen-specific antibodies with clinical potential. Future cloning and expression of the CSPG 4 antigen will allow screening of antibody libraries by means of Phage Display as an alternative method to antibody discovery (Hoogenboom, 2005). Should these investigations help discover novel antibodies with specificity for melanoma antigens, they may reveal new therapeutic options for future effective antibody therapies of melanoma.

In conclusion, we have developed a useful method for one-step antibody cloning in a single mammalian expression vector, allowing the reproducible generation of fully functional recombinant antibodies of any species and isotype with any desired specificity, at the level of tens of milligrams per litre. This method enables the direct comparison of different antibody isotypes that could readily be adapted for use in

mechanistic studies of antigen-antibody interactions, and facilitates the parallel processing of a large panel of antibodies for identification of potential candidates for clinical applications. This is particularly advantageous for expression of whole antibodies derived from Phage Display screening of antibody libraries (Hoogenboom, 2005).

The system is recognised as an unbiased manufacturing platform in academia and is now being applied to other projects, including ovarian cancer antigen, grass pollen allergen and peanut allergen specific antibodies. In addition to all the antibodies characterised in this thesis, the antibody cloning and expression system has enable the generation of all the constructs present in Table 7.1 as well as the cloning, production and characterisation of 7 allergen-specific human isotypes (Figure 7.1). Further reconstruction of the antibody expression cassette into the Flp-In^{TM} system (Invitrogen) for the generation of stable cell lines will provide a number of advantages. An integration of the FRT site into Flp-In^{TM} host cell line will permit polyclonal selection and allows subsequent generation of isogenic stable cell line expressing the antibody of interest much faster and more efficiently.

Isotype / Species	IgE	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2
Human	IgE/κ/λ	IgG1/κ/λ	IgG2/λ	IgG3/λ	IgG4/κ/λ	IgGA1/λ	IgA2/λ
Mouse	IgE/λ	IgG1/κ	–	–	–	–	–
Rat	IgE/κ	–	IgG2b/κ	–	–	–	–

Table 7.1: Antibody expression vectors. pVITRO1 antibody expression vectors generated by PIPE cloning method. Apart from the murine IgG1/k, all other constructs have been used for antibody expression and characterisation. The rat antibodies are used for comparative functional studies in rat xenograft models of human ovarian cancer.

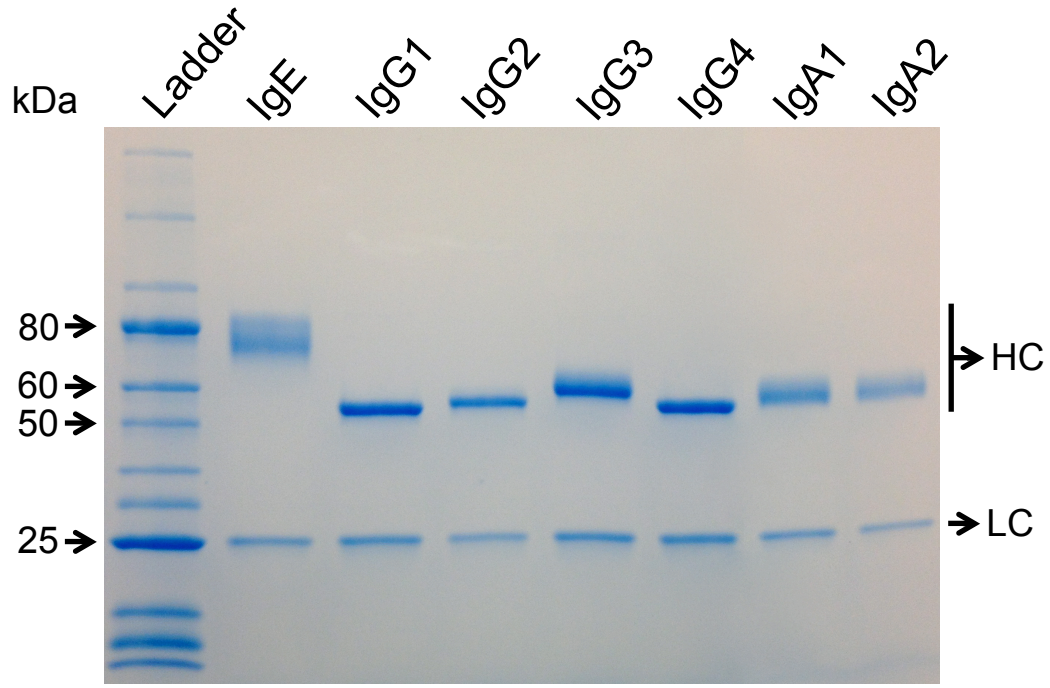


Figure 7.1: SDS-PAGE analysis of Phl p 7 specific recombinant antibodies. Grass pollen allergen Phl p 7 specific antibody variable regions (James et al, 2012) have been cloned in 7 different isotype constructs within a single day using the PIPE cloning method and produced by the stable antibody expression system. Affinity purified antibodies were analysed under reducing conditions and visualised by Coomassie staining.

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